

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/82, 5/10, C07K 16/16, G01N 33/50, A01H 5/00	A2	(11) International Publication Number: WO 00/29592 (43) International Publication Date: 25 May 2000 (25.05.00)
---	-----------	--

(21) International Application Number: PCT/EP99/08710

(22) International Filing Date: 12 November 1999 (12.11.99)

(30) Priority Data:
98 12 1160.0 12 November 1998 (12.11.98) EP
99 11 6981.4 27 August 1999 (27.08.99) EP

(71) Applicant (for all designated States except US):
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG
DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KIRSCH, Christoph [DE/DE]; Am Schulberg 9, D-50858 Köln (DE). LOGE-MANN, Elke [DE/DE]; An der Fuchskaul 2, D-50259 Pulheim-Dansweiler (DE). HAHNBROCK, Klaus [DE/DE]; Bachstrasse 8a, D-50858 Köln (DE). RUSHTON, Paul, [GB/GB]; 24 Poplar Road, Botley, Oxford OX2 9LB (GB). SOMSSICH, Imre [US/DE]; Kolibriweg 2, D-50829 Köln (DE).

(74) Agent: VOSSIUS & PARTNER; Siebertstrasse 4, D-81675 München (DE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: CHIMERIC PROMOTERS CAPABLE OF MEDIATING GENE EXPRESSION IN PLANTS UPON PATHOGEN INFECTION AND USES THEREOF

(57) Abstract

Described are synthetic promoters capable of mediating gene expression in plants upon pathogen infection. Furthermore, recombinant genes and vectors comprising said chimeric promoters as well as host cells transformed with such chimeric promoters, recombinant genes or vectors are provided. Additionally, diagnostic compositions and kits comprising such chimeric promoters, recombinant genes, vectors or cells are described. Provided are further methods for the identification of compounds being capable of activating or inhibiting genes that are specifically expressed in plants upon pathogen infection employing the above described means. Furthermore, transgenic plant cells, plant tissue and plants containing the above-described chimeric promoters, recombinant genes and vectors as well as the use of the aforementioned chimeric promoters, recombinant genes, vectors and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

CHIMERIC PROMOTERS CAPABLE OF MEDIATING GENE EXPRESSION IN PLANTS UPON PATHOGEN INFECTION AND USES THEREOF

The present invention relates to synthetic promoters capable of mediating gene expression in plants upon pathogen infection. The present invention also relates to recombinant genes and vectors comprising said chimeric promoters as well as to host cells transformed with such chimeric promoters, recombinant genes or vectors. The present invention additionally relates to diagnostic compositions and kits comprising such chimeric promoters, recombinant genes, vectors or cells.

The present invention also relates to methods for the identification of compounds being capable of activating or inhibiting genes that are specifically expressed in plants upon pathogen infection employing the above described means. Furthermore, the present invention relates to transgenic plant cells, plant tissue and plants containing the above-described chimeric promoters, recombinant genes and vectors as well as to the use of the aforementioned chimeric promoters, recombinant genes, vectors and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture.

The engineering of disease resistance in crops is a major focus of plant biotechnology. One of the most promising approaches to this problem is to engineer defense reactions that are closely related to natural defense mechanisms such as hypersensitive cell death at infection sites, where the cells immediately surrounding an infection site die in order to prevent further spread of the pathogen (Strittmatter, Bio/Technology 13 (1995), 1085-1089). The controlled generation of highly localized necrotic lesions depends, however, on restricting any cytotoxic activity to the infection sites. This therefore requires promoters that are rapidly and locally responsive to pathogen attack but that also show negligible activity in uninfected tissues.

Initial attempts using large promoter fragments from pathogenesis-related genes such as *prp1-1* have suffered from the disadvantage that it is difficult to isolate a promoter that is totally pathogen specific with substantially no activity in non-infected

tissue (Strittmatter, 1995). It seems likely therefore that very few, if any, naturally occurring promoters will be suitable for this purpose.

Recent advances in the detailed study of defense related genes have identified a number of functionally defined *cis*-acting regulatory DNA elements within pathogen inducible promoters (Korfhage, *The Plant Cell* 6 (1994), 695-708, Raventos, *Plant J.* 7 (1995), 147-155, Rushton, *EMBO J.* 15 (1996), 5690-5700). A number of *cis*-acting elements that are necessary for the response to pathogens have been defined. These include Boxes P and L from the parsley PAL genes (Logemann, *Proc. Natl. Acad. Sci. USA* 92 (1995), 5905-5909), Boxes H and G from soybean PAL and 4CL (Loake, *Proc. Natl. Acad. Sci. USA* 89 (1992), 9230-9234), together with a number of less well defined elements. However, while it was shown for a number of such *cis*-acting elements that they are necessary for elicitor inducibility it was not known whether these elements are sufficient to direct pathogen-induced expression in plant cells and plants on their own. Recently, it has only been shown for the Box W1 from parsley (Rushton, *EMBO J.* 15 (1996), 5690-5700) and ERE from the maize *Prms* (Raventos, *Plant J.* 7 (1995), 147-155) that four copies of these elements alone are sufficient to direct elicitor responsive expression to some extent in transient gene expression assays. However, inducibility and background level of expression of the constructs investigated in Rushton, 1996 and Raventos, 1995 greatly varied and at best an about 10-fold induction of reporter gene expression was observed that may not be sufficient to supply the above-described biotechnological needs. Accordingly, it was unclear whether these or any other *cis*-acting elements may be useful to specifically suppress or confer local gene expression in plants upon pathogen infection.

Thus, the technical problem of the present invention is to provide promoters that are rapidly and locally responsive to pathogen attack but show negligible activity in uninfected parts of the plant and that can be used for engineering of disease resistant crops.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a chimeric promoter capable of mediating local gene expression in plants upon pathogen infection comprising

- (i) at least one *cis*-acting element sufficient to direct elicitor-specific expression comprising the nucleotide sequence of any one of SEQ ID NOS: 3 to 16, and
- (ii) a minimal promoter.

The term "capable of mediating local gene expression in plants upon pathogen infection" as used herein means that said promoter is capable of controlling the expression of a heterologous DNA sequence at infection sites, analogous or closely related to the controlled expression of pathogen related genes which are involved in the natural resistance in most incompatible host/pathogen interactions, such as the hypersensitive cell death at infection sites of a part of a plant. Thus, the chimeric promoter of the invention is characterized by its capability of mediating localized transcriptional activation selectively in response to pathogen attack or in response to stimuli that mimic pathogen attack such as elicitors prepared from, e.g., pathogens such as fungi or bacteria or derivatives thereof. The transcriptional activation by the chimeric promoter of the invention may also occur in cells surrounding the actual infection site due to cell-cell interactions. The chimeric promoter of the invention may advantageously not or only to a small extent be inducible upon other stimuli such as abiotic stress. Preferably, the induction from the chimeric promoter upon pathogen attack or elicitor treatment is at least about 10-fold higher, preferably 20-fold higher and particularly 30-fold higher than its activation, if any, by abiotic stress.

However, the expression specificity conferred by the chimeric promoters of the invention may not be limited to local gene expression due to pathogens, for example, they may be combined with further regulatory sequences that provide for tissue specific gene expression. The particular expression pattern may also depend on the plant/vector system employed. However, expression of heterologous DNA sequences driven by the chimeric promoters of the invention predominantly occurs upon pathon infection or treatment with a corresponding elicitor unless certain elements of the invention were taken and designed by the person skilled in the art to control the expression of a heterologous DNA sequence certain cell types.

The term "*cis*-acting element sufficient to direct elicitor-specific expression" denotes a short stretch of a DNA preferably between 6 and 35 nucleotides in length that when combined with a minimal promoter such as the CaMV 35S minimal promoter

(positions -46 to +8) is capable of directing high level elicitor-specific expression of a heterologous DNA sequence. Preferably, said elicitor is a fungal elicitor that can be prepared by conventional means; see, e.g., Ayers, Plant Physiol. 57 (1976), 760-765; Grosskopf, J. Plant Physiol. 138 (1991), 741-746; Kombrink, Plant Physiol. 81 (1986), 216-221; West, Naturwissenschaften 68 (1981), 447-457.

The term "minimal promoter", within the meaning of the present invention refers to nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and may also include, for example, the TATA box.

The term "pathogen" includes, for example, bacteria, viruses, fungi and protozoa as well as elicitors prepared therefrom.

In accordance with the present invention a number of *cis*-acting elements have been identified that alone are sufficient to direct high level fungal elicitor-specific expression and that can be used to construct novel synthetic promoters that for the first time meet the requirements for engineering disease resistant crops.

Studies that have been performed in accordance with the present invention employed a homologous transient expression system that uses parsley (*Petroselinum crispum*) protoplasts derived from cultured cells. This system is one of very few where the protoplasts respond to fungal elicitor molecules in an almost identical way to cells in the intact plant (Dangl, EMBO J. 6 (1987), 2551-2556; Hahlbrock, Proc. Natl. Acad. Sci. USA 92 (1995), 4150-4157). This allows the study of elicitor-responsive *cis*-acting elements, something that is difficult in many other experimental systems.

Eleven *cis*-acting elicitor-responsive elements (SEQ ID NOS: 3 to 13) were identified in accordance with the present invention. Monomers and multimers of each element were constructed in addition to synthetic promoters consisting of two or more of these elements in combination. Each construct was synthesized with either BamHI ends or with a SpeI site at the 5' end and an XbaI site at the 3' end and then cloned into the corresponding restriction site in front of a minimal CaMV 35S promoter (-46 to +8) in the vector MS23-pBT10-GUS (Sprenger, Ph.D. thesis, University of Köln, Köln, Germany (1997); see Figure 1 (SEQ ID NO: 17) and Figure 2). The distance between the insertion site and the TATA Box varied between 25 and 70 bp

depending on the insertion site employed and only slight differences, if any, were seen when the same element was inserted into different restriction sites.

Additionally, the *cis*-acting elicitor-responsive element Box E17 (SEQ ID NO: 15) was identified in accordance with the present invention. Synthetic promoters were constructed comprising a monomer, a dimer or the reverse complement of this element. Various distances between 5 and 131 bp from the inserted Box E17 to the minimal promoter were tested using monomers and dimers (see Example 7). Usable inducibility in the sense of the present invention was obtained for distances of at least 12 bp, and optimal inducibility for distances of 40 to 60 bp to the 5'-end of the minimal promoter. Another *cis*-acting element of the present invention, the 21 bp long 3'-fragment of Box E17 (SEQ ID NO: 16) confers a similar elicitor-responsiveness as compared to Box E17 (see Example 6).

The experiments performed in accordance with the present invention demonstrate that the *cis*-acting elements direct pathogen-induced expression *in vivo*, being active as monomers, multimers and in combination with each other within synthetic promoters. They therefore meet the biotechnological requirements for the engineering of disease resistance.

In accordance with the present invention these novel chimeric promoters cloned in front of the GUS coding region and the resulting chimeric genes were introduced by means of vacuum infiltration mediated gene transfer into *Arabidopsis* plants; see Example 8. The expression pattern observed in the transgenic plants containing the GUS marker gene under the control of the chimeric promoter of the invention revealed expression in tissue infected by bacterial (*Pseudomonas syringae*) as well as by fungal pathogens (*Peronospora parasitica*), whereas local expression in wounded tissues seems to be inactive.

The chimeric promoter of the invention may be preferably comprised only of the above defined *cis*-acting elements and a minimal promoter. As will be discussed below, other regulatory sequences may be added or present dependent on the intended use of the chimeric promoter of the invention. However, preferably the chimeric promoter of the invention lacks elements that interfere with the elicitor

specific expression and/or which are responsible for the non-selective expression of the promoter the *cis*-acting element of the invention was derived from.

To obtain possible expression in all tissues of a transgenic plant, the minimal regulatory sequences of constitutive promoters are often used, such as the 35 S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). It is also immediately evident to the person skilled in the art that further regulatory elements may be added to the chimeric sequences of the invention. For example, transcriptional enhancers and/or sequences which allow for further induced expression of the chimeric promoter of the invention may be employed. Enhancer sequences functional in plants include, for example, ocs-element (Ellis, EMBO J. 6 (1987), 3203-3208); the family of ACGT-elements (hex-motif, G-box as 1-element) (Williams, Plant Cell 4 (1992), 485-496) and the cyt-1 element (Neuteboom, Plant J. 4 (1993), 525-534). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, *Vicia*, wheat, barley etc. Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366).

Preferably, the chimeric promoter of the invention further comprises a *cis*-acting element having the nucleotide sequence of SEQ ID NO: 1 or 2; see Example 5.

In a particularly preferred embodiment of the invention the chimeric promoter comprises homo- and/or hetero-multimeric forms of said *cis*-acting element(s); see also the appended Example 5. Preferably, said multimeric form is a dimer or tetramer. Particularly preferred are those combinations of *cis*-acting elements that are described in Example 5 and which combination provide for an at least 20-fold, preferably at least 30-fold and particularly preferred at least about 50-fold induction.

In a preferred embodiment of the chimeric promoter of the invention the minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, or

hcbt2 promoter. However, other minimal promoters from other sources may be employed as well.

In a further preferred embodiment of the chimeric promoter of the invention, the distance between said *cis*-acting element and said minimal promoter is 12 to 300 base pairs, more preferably 25 to 70 base pairs, and most preferably 40 to 60 base pairs. In addition or alternatively, a spacer region preferably composed of 4 to 10 base pairs separates at least two of said *cis*-acting elements in the chimeric promoter. Likewise, it is preferred that at least two of said multimeric forms in the chimeric promoter described above are separated by a spacer of between about 50 to 1000 base pairs.

In a particularly preferred embodiment of the chimeric promoter of the invention the induction of gene expression upon elicitor treatment or pathogen infection is at least 15-fold. As discussed before, the *cis*-acting elements so far investigated in the prior art only provided for induction upon elicitor treatment of about 10-fold. However, a 10-fold induction of a recombinant gene encoding, e.g., an anti-viral protein may not be sufficient to rapidly and efficiently combat against the pathogen. The present invention provides several *cis*-acting elements that are capable of inducing high level expression of a given DNA sequence up to 400-fold induction; see, e.g., Example 1. Furthermore, the invention demonstrates that the combination of otherwise weak *cis*-acting elements can provide for a substantial increase of the overall inducibility of the chimeric promoter; see Example 5. Thus, the present invention for the first time provides a generally applicable method for how to construct and use chimeric promoters in the field of plant biotechnology. As will be noted from the appended Examples, the background value of the chimeric promoters of the invention may vary to a certain extent. The person skilled in the art therefore may employ different chimeric promoters with different background levels and inducibility depending on the intended use. For example, if the approach of coat protein-mediated protection against virus infection is used the chimeric promoter employed may have high background level expression that would not harm the plant and which upon viral infection would increase at high levels such that resistance to the virus can be obtained. The same rationale would apply to, e.g., an antisense or ribozyme mediated protection or the engineering of resistance to fungal pathogens by the expression of

anti-fungal proteins etc. On the other hand, where the generation of race-specific resistant genes and artificial generation of hypersensitive cell death is intended, preferably a chimeric promoter is used that has low or substantially no background activity and that only upon pathogen attack is activated to an extent that sufficient level of toxic protein is made so as to cause the cell to die. The selection of the appropriate chimeric promoter of the invention depending on its use is well within the skill of the person skilled in the art.

Examples of the different possible applications of the chimeric promoter according to the invention as well as its *cis*-acting elements will be described in detail in the following.

Hence, in a further embodiment, the present invention relates to a recombinant gene comprising the above-described chimeric promoter. Preferably, the recombinant gene is configured such that the chimeric promoter is operatively linked to a heterologous DNA sequence.

The term "heterologous" with respect to the DNA sequence being operatively linked to the chimeric promoter of the invention means that said DNA sequence is not naturally linked to the chimeric promoter of the invention.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. The chimeric promoter "operably linked" to a heterologous DNA sequence is ligated in such a way that expression of a coding sequence is achieved under conditions compatible with the control sequences. Expression comprises transcription of the heterologous DNA sequence preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic, i.e. plant cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV) and the Nopaline Synthase gene from *Agrobacterium tumefaciens*. Additional regulatory elements may include transcriptional as well as translational enhancers. A plant translational enhancer often used is the CAMV omega sequences, the inclusion of

an intron (Intron-1 from the Shrunk gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). In this respect, it should be noted that in one embodiment of the recombinant gene of the invention at least one of said *cis*-acting elements is located in the 5'- or 3'-untranslated region or in an intron of the recombinant gene.

In a preferred embodiment of the recombinant gene of the invention said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme, transcription factor, protease, nuclease, lipase, or polymerase

The recombinant gene of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins for, e.g., the control of disease resistance or diagnostics of pathogen inducible or related gene expression. The recombinant gene or vector containing the DNA sequence encoding an RNA or a protein of interest is introduced into the cells which in turn produce the RNA or protein of interest. For example, the chimeric promoter of the invention can be operatively linked to DNA sequences encoding Barnase for use in the production of localized cell death in plants upon pathogen attack.

On the other hand, said protein can be a scorable marker, e.g., luciferase, green fluorescent protein or β -galactosidase. This embodiment is particularly useful for simple and rapid screening methods for compounds and substances described herein below capable of modulating pathogen specific or elicitor inducible gene expression. For example, transgenic plant cells can be cultured in the presence and absence of a candidate compound in order to determine whether the compound affects the expression of genes which are under the control of chimeric promoters of the invention, which can be measured, e.g., by monitoring the expression of the above-mentioned marker. It is also immediately evident to those skilled in the art that other marker genes may be employed as well, encoding, for example, a selectable marker which provides for the direct selection of compounds which induce or inhibit the expression of said marker.

The chimeric promoters of the invention may also be used in methods of antisense approaches. The antisense RNA may be a short (generally at least 10, preferably at

least 14 nucleotides, and optionally up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence and/or DNA sequence of the gene of interest. Standard methods relating to antisense technology have been described; see, e.g., Klann, *Plant Physiol.* 112 (1996), 1321-1330. Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target sequence within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA.

Furthermore, appropriate ribozymes can be employed (see, e.g., EP-A1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a target gene. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, *Ribozymes, Methods in Cell Biology* 50, Galbraith, eds Academic Press, Inc. (1995), 449-460. Further applications of the chimeric promoter are evident to the person skilled in the art and can be derived from the literature, e.g., Strittmatter and Wegener, *Zeitschrift für Naturforschung* 48c (1993), 673-688; Kahl, *J. Microbiol. Biotechnol.* 11 (1995), 449-460 and references cited therein.

Said transcription factor can for example be a master regulatory factor that controls the expression of a cascade of genes involved in pathogen defense of the plant (Grotewold, *Plant Cell* 10 (1998), 721-740; Rushton and Somssich, *Curr. Opin. Plant Biol.* 1 (1998), 311-315). Alternatively, it can be a hybrid transcription factor containing a DNA-binding domain (e.g. of GAL4 or of the bacteriophage 434) and an activator domain (e.g. of VP16 or of any functional plant activator domain), which, when expressed in transgenic plants containing an antisense target gene under the control of a synthetic promoter containing the appropriate *cis*-acting element recognizing the hybrid factor, leads to specific repression (knock-out) of the desired endogenous gene function (Wilde, *Plant Mol. Biol.* 24 (1994), 381-388; Guyer, *Genetics* 149 (1998), 633-639).

Suitable lipases comprise for example phospholipases, e.g., C or A₂ type phospholipases (Scherer, *Plant Growth regulation* 18 (1996), 125-133). Lipases are capable of releasing free fatty acids from membrane lipids, wherein these fatty acids can function as signal transducers by which general cellular defense reactions are

elicited. The growing importance of free fatty acids in pathogen-defense is documented, e.g., in Scherer (1996), Roy (Plant Sci. 107 (1995), 17-25 and references cited therein) and Tavernier (Plant Sci. 104 (1995), 117-125).

Nucleases, i.e. RNases and DNases, may also be employed, of which Barnase is one candidate among others. The use of proteases in the context of this embodiment may apply to cytotoxic effects.

A signal amplification system may be constructed using polymerases. In a two-step model, an elicitor-induced polymerase, e.g., SP6-, T7- or T3-RNA polymerase, can transcribe a second recombinant gene which is controlled by a promoter to which the polymerase is highly specific. The second gene may encode for example a cytotoxic protein which is then expressed in an amplified way. A plant system based on T7-RNA polymerase was described by McBride (Proc. Natl. Acad. Sci. USA 91 (1994), 7301-7305).

Cytotoxic proteins comprise, for example, plant RIPs (ribosome inactivating proteins; (Stripe, Bio/Technology 10 (1992), 405-412), defensins (Broekaert, Plant Physiol. 108 (1995), 1353-1358), Bt toxin, α -amylase inhibitor, T4-lysozyme, avirulence gene products, or enzymes such as glucose oxidase which generate reactive oxygen species (Shah, Trends Biotechnol. 13 (1995), 362-368; Shah, Curr. Opin. Biotech. 8 (1997), 208-214; Beachy, Curr. Opin. Biotech. 8 (1997), 215-220; Cornelissen, Plant Physiol. 101 (1993), 709-712; Estruch, Nucleic Acids Res. 22 (1994), 3983-3989).

It is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the nucleus, endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art. (Görlisch, Science 271 (1996), 1513-1518; Hicks, Plant Physiol. 107 (1995), 1055-1058; Rachubinski, Cell 83 (1995), 525-528; Schatz, Science 271 (1996), 1519-1526; Schnell, Cell 83 (1995), 521-524; Verner, Science 241 (1988), 1307-1313; Vitale, BioEssays 14 (1992), 151-160).

The present invention also relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a

chimeric promoter or a recombinant gene of the invention. Preferably, said vector is a plant expression vector, preferably further comprising a selection marker for plants. For example of suitable selector markers, see *supra*. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the chimeric promoters and recombinant genes of the invention can be reconstituted into liposomes for delivery to target cells.

Advantageously, the above-described vectors of the invention comprise a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, *Plant Physiol. (Life Sci. Adv.)* 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, *EMBO J.* 2 (1983), 987-995) and hygromycin (Marsh, *Gene* 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, *Proc. Natl. Acad. Sci. USA* 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory ed.) or deaminase from *Aspergillus terreus* which confers resistance to Blasticidin S (Tamura, *Biosci. Biotechnol. Biochem.* 59 (1995), 2336-2338).

Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, *Pl. Sci.* 116 (1996), 59-72; Scikantha, *J. Bact.* 178 (1996), 121), green fluorescent protein (Gerdes, *FEBS Lett.* 389 (1996), 44-47) or β -glucuronidase (Jefferson, *EMBO J.* 6 (1987), 3901-3907). This embodiment is particularly useful for

simple and rapid screening of cells, tissues and plants containing a vector of the invention.

The present invention furthermore relates to host cells comprising a chimeric promoter, recombinant gene or a vector according to the invention wherein the chimeric promoter is foreign to the host cell.

By "foreign" it is meant that the chimeric promoter is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said *cis*-acting element. This means that, if the *cis*-acting element is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. The vector or recombinant gene according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally. In this respect, it is also to be understood that the chimeric promoter or recombinant gene of the invention can be used to restore or create a mutant gene via homologous recombination (Paszkowski (ed.), Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers (1994)). The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred cells are plant cells.

In a further preferred embodiment, the present invention provides for a method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a chimeric promoter, recombinant gene or vector of the invention into the genome of said plant, plant cell or plant tissue. For the expression of the heterologous DNA sequence under the control of the chimeric promoter according to the invention in plant cells, further regulatory sequences such as poly A tail may be fused, preferably 3' to the heterologous DNA sequence, see also supra. Further possibilities might be to add Matrix Attachment Sites at the borders of the transgene to act as "delimiters" and insulate against methylation spread from nearby heterochromatic sequences.

Methods for the introduction of foreign genes into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA

using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, vacuum infiltration, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of *Agrobacterium* which allow stable integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361; Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of *Agrobacterium tumefaciens* and vectors as well as transformation of *Agrobacteria* and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasterdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of *Agrobacterium tumefaciens* is preferred in the method of the invention, other

Agrobacterium strains, such as *Agrobacterium rhizogenes*, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, *Plant Physiol.* 104 (1994), 37-48; Vasil, *Bio/Technology* 11 (1993), 1553-1558 and Christou (1996) *Trends in Plant Science* 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), *Gene Transfer To Plants*. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.

The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person.

Alternatively, a plant cell can be used and modified such that said plant cell expresses an endogenous gene under the control of the chimeric promoter. The introduction of the chimeric promoter of the invention which does not naturally control the expression of a given gene or genomic sequences using, e.g., gene targeting vectors can be done according to standard methods, see *supra* and, e.g., Hayashi, *Science* 258 (1992), 1350-1353; Fritze and Walden, *Gene activation by T-DNA tagging*. In *Methods in Molecular biology* 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, *Physiologia Plantarum* 78 (1990), 105-115).

In general, the plants which can be modified according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells comprising, preferably stably integrated into the genome, a chimeric promoter, a recombinant gene or vector according to the invention or obtainable by the above-described method.

Furthermore, the present invention also relates to transgenic plants and plant tissue comprising the above-described transgenic plant cells or obtainable by the above-described method. These plants may show, for example, increased disease resistance.

In a preferred embodiment of the invention, the transgenic plant upon the presence of the chimeric promoter or the recombinant gene of the invention attained resistance or improved resistance against a pathogen the corresponding wild-type plant was susceptible to.

The term "resistance" covers the range of protection from a delay to complete inhibition of disease development. Examples for pathogens of importance comprise *Phytophthora infestans*, the causal agent of potato late blight disease, *Phytophthora sojae*, root rot pathogen of soybean, *Peronospora parasitica* (downy mildew), *Magnaporthe grisea*, causal agent of rice blast disease, *Erysiphe* spp (powdery mildew), *Pseudomonas syringae* (agent of bacterial blight), *Erwinia amylovora* (fire blight disease), *Erwinia carotovora* (soft rot), *Botrytis cinerea* (downy mildew of grape), *Rhizoctonia solani* and *Pythium debaryanum* (agents of seedling blight or damping off disease).

In yet another aspect the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells described above. Harvestable parts can be in principle any useful part of a plant, for example, leaves, stems, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

As discussed above, novel *cis*-acting elements have been identified in accordance with the present invention that are capable of conferring elicitor inducible or pathogen

specific gene expression in plant cells and plants. Therefore, the present invention also relates to *cis*-acting elements as defined above or multimeric forms of any one of those as discussed hereinbefore.

Due to the tight regulation of the chimeric promoters of the invention it is evident that they are particularly suited for the identification of compounds that either specifically interact with these *cis*-acting elements or that act upstream of the signal transduction pathway that leads to activation of genes the *cis*-acting elements were derived from.

Thus, the present invention further relates to a method for the identification of an activator or inhibitor of genes specifically expressed in plants upon pathogen infection comprising the steps of:

- (a) providing a plant, plant cell, or plant tissue comprising a recombinant DNA molecule comprising a readout system operatively linked to the chimeric promoter of the invention;
- (b) culturing said plant cell or tissue or maintaining said plant in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said readout system;
- (c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or plant tissue.

For the identification of inhibitors, it is advantageous to include an elicitor or another activator known to be capable of inducing the activity of promoters that contain the *cis*-acting elements of the chimeric promoters of the invention in step (b) of the above-described method, and to determine whether the compound to be screened suppresses the induction of the readout system by said elicitor or activator.

The term "readout system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such readout systems are well known to those skilled in the art and comprise, for example, recombinant genes and marker genes as described above and in the appended examples.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.

Said compound or plurality of compounds may be comprised in, for example, samples of inorganic or organic molecules or, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating pathogen related genes. Suitable set ups for the method of the invention are known to the person skilled in the art. The plurality of compounds may be, e.g., added to the cell or tissue culture medium or soil, injected into the cell or sprayed onto the plant.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating the chimeric promoter of the invention, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above described method or its analog or derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture. For example, it can be combined with a agriculturally acceptable carrier known in the art.

The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, *Nature Medicine* 1 (1995), 879-880; Hupp, *Cell* 83 (1995), 237-245; Gibbs, *Cell* 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of genes controlled by the *cis*-acting elements of the invention and/or which exert their effects up- or downstream from such genes may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art (see, e.g., Hayashi, *Science* 258 (1992), 1350-1353; Fritze and Walden, *Gene activation by T-DNA tagging*. In *Methods in Molecular biology* 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa:

Human Press (1995), 281-294) or transposon tagging (Chandlee, *Physiologia Plantarum* 78 (1990), 105-115).

Said compounds can also be functional derivatives or analogues of known inhibitors or activators. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, *Handbook of Organic Chemistry*, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and *Organic Synthesis*, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art or as described in the appended examples. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used. The cell or tissue that may be employed in the method of the invention preferably is a plant cell, plant tissue or plant of the invention described in the embodiments hereinbefore.

In an additional embodiment, the characteristics of a given compound may be compared to that of a cell contacted with a compound which is either known to be capable or incapable of suppressing or activating the chimeric promoter of the invention or the promoter the *cis*-acting element of the chimeric promoter is derived from.

The inhibitor or activator identified by the above-described method may prove useful as a plant protective agent or herbicide or pesticide. Thus, in a further embodiment the invention relates to a compound obtained or identified according to the method of the invention said compound being an activator or an inhibitor of genes specifically induced upon pathogen infection.

Furthermore, identification of *trans*-acting factors which interact with the *cis*-acting elements of the invention can form the basis for the development of novel agents for modulating conditions associated with plant diseases. Identification of *trans*-acting factors is carried out using standard methods in the art (see, e.g., Sambrook, *supra*, and Ausubel, *supra*). To determine whether a protein binds to the *cis*-acting elements of the invention standard DNA footprinting and/or native gel-shift analyses can be carried out. In order to identify the *trans*-acting factor which binds to the *cis*-acting elements of the invention, these elements can be used as an affinity reagent

in standard protein purification methods, or as a probe for screening an expression library. Once the *trans*-acting factor is identified, modulation of its binding to the *cis*-acting elements of the invention can be pursued, beginning with, for example, screening for inhibitors of *trans*-acting factor binding.

Activation or repression of genes involved in plant defense reactions could then be achieved in plants by applying of the *trans*-acting factor (or its inhibitor) or the gene encoding it, e.g. in a vector for transgenic plants. In addition, if the active form of the *trans*-acting factor is a dimer, dominant-negative mutants of the *trans*-acting factor could be made in order to inhibit its activity. Furthermore, upon identification of the *trans*-acting factor, further components in the pathway leading to activation (e.g. signal transduction) or repression of pathogenesis related genes then can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional agents and methods for modulating the response of plants upon pathogen attack in plants.

Accordingly, the present invention also relates to a plant protection composition comprising the compound identified and obtained by the above described methods. The plant protection composition can be prepared by employing the above-described method of the invention and synthesizing the compound identified as inhibitor or activator in an amount sufficient for use in agriculture. Thus, the present invention also relates to a method for the preparation of an agricultural plant protection composition comprising the above-described steps of the method of the invention and synthesizing the compound so identified or an analog or derivative thereof.

In the plant protection composition of the invention, the compound identified by the above-described method may be preferentially formulated by conventional means commonly used for the application of, for example, herbicides and pesticides or agents capable of inducing systemic acquired resistance (SAR). For example, certain additives known to those skilled in the art stabilizers or substances which facilitate the uptake by the plant cell, plant tissue or plant may be used, for example, harpins, elicitors, salicylic acid (SA), benzol(1,2,3)thiadiazole-7-carbothioic acid (BTH), 2,6-dichloro isonicotinic acid (INA), jasmonic acid (JA), methyljasmonate.

In a further embodiment, the present invention relates to an antibody specifically recognizing the compound obtainable by the method of the invention or the *cis*-

acting element described above. The antibodies of the invention can be used to identify and isolate other activators and inhibitors of genes that are involved in plant defense. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, *Nature* 256 (1975), 495, and Galfré, *Meth. Enzymol.* 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988.

Furthermore, the present invention relates to a diagnostic composition comprising the chimeric promoter, the recombinant gene, the vector, the compound or the antibody of the invention, and optionally suitable means for detection. Said diagnostic compositions may be used for, e.g., methods for screening activators or inhibitors as described above.

In addition, the present invention relates to a kit comprising the chimeric promoter, the recombinant gene, the vector, the compound or the antibody of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. Furthermore, the kit may include buffers and substrates for reporter genes that may be present in the recombinant gene or vector of the invention. In addition, the kit of the invention may contain compounds such as elicitors, preferably fungal elicitors that can be used as standards for the expression assays. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to herein, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art.

The kit or its ingredients according to the invention can be used in plant cell and plant tissue cultures, for example, for any of the above described methods for

detecting inhibitors and activators of pathogenesis related genes. The kit of the invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as disease resistance.

It is also immediately evident to the person skilled in the art that the chimeric promoters, recombinant genes and vectors of the present invention can be employed to produce transgenic plants with a desired trait (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397) comprising (i) insect resistance (Vaek, Plant Cell 5 (1987), 159-169), (ii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology & Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996), 56 suppl.), (iii) resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141), (iv) inducing and maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695) or may be used as highly inducible production systems of heterologous proteins or biopolymers in plants analogous to inducible systems in bacteria.

The present invention for the first time demonstrates that a number of *cis*-acting elements that are responsible for inducibility of pathogenesis-related genes can be used either alone or in combination with themselves or with other *cis*-acting elements to construct chimeric promoters that are capable of mediating highly inducible gene expression in plant cells upon elicitor treatment. It is therefore evident that *cis*-acting elements derived, e.g., from pathogen-related promoters other than those specifically described above can be used in accordance with the present invention, for example, chitinase promoters; see, e.g., Kellmann, Plant. Mol. Biol. 30 (1996), 351-358. Appropriate promoters that provide a source for such *cis*-acting elements can be used and obtained from any plant species, for example, maize, potato, sorghum, millet, coix, barley, wheat and rice. Such promoters are characterized by their inducibility upon pathogen infection.

For example, using cDNA of proteins that are specifically expressed in plants upon pathogen attack as probes, a genomic library consisting of plant genomic DNA cloned into phage or bacterial vectors can be screened by a person skilled in the art.

Such a library consists, e.g., of genomic DNA prepared from plant leaf tissue, fractionized in fragments ranging from 5 kb to 50 kb, cloned into the lambda vectors such as Lambda EMBL3 or 4, Lambda ZAP, Lambda DASH or Lambda GEM. Phages hybridizing with the probes can be purified. From the purified phages DNA can be extracted and sequenced. Having isolated the genomic sequences corresponding to the genes encoding the PR proteins, it is possible to fuse heterologous DNA sequences to these promoters or their regulatory sequences via transcriptional or translational fusions according to methods well known to the person skilled in the art. In order to identify the regulatory sequences and specific elements of these genes, 5'-upstream genomic fragments can be cloned in front of marker genes such as *luc*, *gfp* or the GUS coding region and the resulting chimeric genes can be introduced by means of *Agrobacterium tumefaciens* mediated gene transfer into plants or transfected into plant cells or plant tissue for transient expression. The expression pattern observed in the transgenic plants or transfected plant cells containing the marker gene under the control of the isolated regulatory sequences reveal the boundaries of the promoter and its *cis*-acting elements. The isolation of *cis*-acting elements having the above defined properties can be done by conventional techniques known in the art, for example, by using DNaseI footprinting and loss-and gain-of-function experiments. It is then possible to isolate the corresponding promoter region by conventional techniques and test it for its expression pattern. For this purpose, it is, for instance, possible to fuse the putative *cis*-acting element with a minimal promoter to a reporter gene, such as GUS, luciferase or green fluorescent protein (GFP) and assess the expression of the reporter gene in transient expression assays or transgenic plants; see also the appended examples.

Thus, the present invention relates to the use of a *cis*-acting element sufficient to direct elicitor-specific expression and in particular to the use of the chimeric promoter, the recombinant gene, the vector, the *cis*-acting element and/or the compound of the present invention for the production of pathogen resistant plants or for identifying and/or producing compounds capable of conferring induced resistance to a pathogen in a plant.

In a still further embodiment, the present invention relates to a method of rendering a gene responsive to pathogens comprising inserting at least one *cis*-acting element sufficient to direct elicitor-specific expression into the promoter of said gene. As is evident to the person skilled in the art a promoter that displays the capabilities of the chimeric promoter of the invention can also be obtained by introducing the *cis*-acting element as defined above into a promoter of a gene, preferably in close proximity to the transcription initiation site of the gene.

In another embodiment, the present invention relates to a method for preparing a promoter capable of mediating local gene expression in plants upon pathogen infection comprising operably linking a *cis*-acting element sufficient to direct elicitor-specific expression to a transcription initiation sequence of a promoter. Preferably, said *cis*-acting element to be inserted in the above-described methods is a *cis*-acting element of the present invention or as defined in the foregoing embodiments or a multimeric form thereof as defined hereinabove. As mentioned before, the elicitor responsive *cis*-acting elements are preferably responsive to fungal elicitor.

In a preferred embodiment of the invention, the above-described methods further comprising deleting non-specific *cis*-acting elements in the promoter. Introduction of the *cis*-acting element of the invention into a given promoter per se may not be sufficient to direct the promoter to exclusively mediate local gene expression in plants upon pathogen infection. In this case, preexisting elements that may be responsive, for example, to light, hormones, low temperatures, drought or salt stress may be deleted.

The above described methods give rise to novel chimeric promoters that are at least partially, preferably fully controlled by plant/pathogen interaction.

Accordingly, the present invention also relates to the promoter obtainable by a method as described above. Said promoter can then be employed for the embodiments described hereinabove.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the

methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under <http://www.ncbi.nlm.nih.gov/PubMed/medline.html>. Further databases and addresses, such as <http://www.ncbi.nlm.nih.gov/>, <http://www.infobiogen.fr/>, http://www.fmi.ch/biology/research_tools.html, <http://www.tigr.org/>, are known to the person skilled in the art and can also be obtained using, e.g., <http://www.lycos.com>. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The present invention is further described by reference to the following non-limiting figures and examples.

The Figures show:

Figure 1 Restriction map of the plasmid ms23 (Sprenger, 1997)

Figure 2 Overview cartoon of the plasmid ms23. The Gus reporter gene and minimal -46 CaMV 35S promoter are shown, as are restriction sites found in the polylinker sequence situated 5' to the minimal promoter. The distances (in base pairs) between the restriction sites are also shown.

Figure 3 Overview cartoon of the plasmid pGPTV. The Gus reporter gene and minimal -46 CaMV 35S promoter are shown as are the *SpeI* and *XbaI* sites used in making the constructs employed. The *nptII* selection marker is also indicated, as are the left and right T-DNA borders (L and R). The terminators (pA_{nos} and p_{nos}) and promoter driving the *nptII* gene (pAg7) are also shown.

Figure 4 Elicitor inducibility of chimeric promoters containing Box E17 and derivatives thereof. GTAC motifs in forward and reverse orientation are

underlined. Deleted bases are depicted as \emptyset . The depicted fragments are located 12 bp upstream of the 35S minimal promoter. The monomers of the dimeric construct A109 are separated by a 6 bp restriction site.

- Figure 5** Elicitor inducibility of chimeric promoters containing dissected Box E17 elements. Starting from a Box E17 containing chimeric promoter (H149), chimeric promoters were constructed having 6 nucleotides deleted from the 5'-end of Box E17 (B175), 7 nucleotides from its 3'-end (C175) or comprising both deletions (C175). Additionally, a promoter was tested comprising a 445 bp Eli17 promoter fragment from which the 27 bp Box Eli17 element was deleted (A175). Relative and absolute elicitor induction values are given that were measured in transient expression assays.
- Figure 6** Cut-out of the polylinker of the vector ms23. For measuring the influence of the distance to the 35S minimal promoter Box E17 or its dimer was inserted into eight different restriction sites.
- Figure 7** Elicitor inducibility of Box E17 depending on the distance to the 35S minimal promoter. The induction upon elicitor treatment is given for the constructs as illustrated in Figure 6. ms23 represents the vector only containing the minimal promoter as negative control.
- Figure 8** Expression characteristics of transgenic plants transformed with reporter gene constructs comprising chimeric promoters with tetramers of some *cis*-elements of the present invention. For comparison the GCC-Box element is included (see Example 1). The background expression levels are quantified as being low (barely detectable background expression), medium (visible background expression but induction by pathogens is clearly visible over the background) or very high (extremely high expression such that induction by pathogens is difficult to detect). A minus indicates no detectable expression, a plus indicates inducible expression and "nt" not tested.

The Examples illustrate the invention:

Experimental setup

1. Recombinant DNA techniques

Unless stated otherwise in the examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook (1989), Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications (UK).

2. Transient expression vector

All constructs, unless a different protocol is given in the examples, were cloned between the SpeI and XbaI sites in pbt10-GUS (ms23) (Sprenger, 1997). At the 3' end of each construct is an intact XbaI site (6 bp) followed immediately by a minimal CaMV 35S promoter (-46 to +8). The 3' end of all inserts are therefore 28 bp upstream of the CaMV TATA Box and 52 bp upstream of the start of transcription. Multiple copies of the elements are separated by 6 base pairs (TCTAGT) created by the ligation of a SpeI sticky end with a XbaI sticky end. The sequence of ms23 (SEQ ID NO: 17) as a restriction map and an overview cartoon are provided (Figures 1 and 2).

3. Transgenic plant vector

The vector employed was pGPTV-GUS-kan (Becker, Plant Mol. Biol. 20 (1992), 1195-1197). The polylinker, minimal CaMV 35S promoter and GUS reporter gene are identical to ms23. All spacings and orders of *cis*-elements within the constructs are therefore identical to those in the corresponding transient expression constructs in ms23. A cartoon of pGPTV is provided (Figure 3).

4. Transient transfection and expression assays

The transient transfection and expression assays were essentially carried out as described in Dangl, EMBO J. 6 (1987), 2551-2556; Schulze-Lefert, EMBO J. 8 (1989), 651-656; van de Löcht, EMBO J. 9 (1990), 2945-2950. Briefly, five day old subcultured parsley cells are used for the isolation of protoplasts. Protoplasting is achieved by overnight incubation of the cells in 0.24 M CaCl₂ containing 0.25% (w/v) cellulase and 0.05% (w/v) macerozyme at 24° C. Protoplasts are collected by centrifugation (7 min., 100 g), washed with 0.24 M CaCl₂, and then floated in B5 medium (GIBCO/BRL) containing 0.4 M sucrose and 1 mg/ml 2,4-dichlorophenoxyacetic acid. Protoplasts floating after centrifugation (5 min, 100 g) were harvested, counted and adjusted to 2×10^6 /ml.

Supercoiled or linearized plasmid DNA (5 -20 μ g) containing the chimeric promoter-reporter(GUS) construct was transferred into the protoplasts using the polyethylene glycol (PEG) method (Krens, Nature 296 (1982), 72-74). Each transformation assay was split and placed into two 3 ml plates. The Pep25 (Nürnberg, Cell 78 (1994), 449-460) elicitor was added to one whereas the other served as a control. Both samples were harvested after 8 hours, frozen in liquid nitrogen, crude protein extracts prepared and GUS activity assayed (Jefferson, Plant Mol. Biol. Rep. 5 (1987), 387-405). Bradford assays (Bio-Rad) were used for protein determination. The expression data are given as mean fold induction values \pm standard deviation (SD) and mean GUS activity (pmol/min/mg) from six independent transient transfection assays treated with or without Pep25 elicitors.

5. Generation of transgenic plants

Transgenic plants were generated according to the methods described in Bechtold, Mol. Biol. Genet. 316 (1993), 1194-1199; Grant, Science 269 (1995), 843-846 and Dangl, Science 269 (1995), 843-846. Briefly, the promoter elements were cloned in front of the reporter gene of the binary vector pGPTV-GUS-kan (Becker, Plant Mol. Biol. 20 (1992), 1195-1197) and the constructs introduced into the *Agrobacterium* strain GV3101 (pMP90; (Koncz and Schell, loc. cit.) containing the pMP90 helper plasmid. 500ml cultures were grown in YEB medium containing kanamycin (50 μ g/ml), rifampicin (100 μ g/ml) and gentamycin (25 μ g/ml). Cells were resuspended in infiltration medium (0.5 x Murashige-Skoog salts; 1 x B5 vitamins; 5.0% sucrose and 0.044 μ M benzlamino purine) and vacuum infiltrated into *Arabidopsis* plants by

the method of Grant (1995). T1 seeds were surface-sterilized and transformants were selected on MS medium containing 50 μ g/ml kanamycin. Primary transformants were transferred to soil and tested for GUS expression during pathogenesis and biotic or abiotic stress.

Example 1: Box S is capable of mediating elicitor induced gene expression

Box S (CAGCCACCAAAGAGGACCCAGAAT; SEQ ID NO: 7) has been shown to be necessary for the elicitor-responsive expression of the parsley *eli 7* genes (Takamiya-Wik, Ph.D. thesis, University of Köln, Köln, Germany (1995)). Together with the results concerning Box N (see Example 4.3) for the first time the core sequence of this type of element has been defined which appears to be AGCCACCANA. The element is not identical to any known elicitor-responsive element although it is very similar to a number of ethylene response elements that have the core sequence AGCCGCC (GCC Boxes) (Ohme-Takagi and Shinshi, The Plant Cell 7 (1995), 173-182). In the promoters investigated (*eli7-1*, *eli7-2* and *Prp1*) there is always an A residue rather than a G. What difference this difference in sequence makes is at present unclear and it is not known whether Box S is responsive to ethylene. It has however been shown for the first time that the Box S elements with the sequence AGCCACC are elicitor-responsive elements. The present data also show for the first time that GCC Boxes are also elicitor response elements as well as being ethylene response elements. Box S is a very strong elicitor-responsive element. A monomer of Box S gives 11-fold inducibility and a tetramer up to 560-fold inducibility. This clearly shows Box S to be an extremely promising element for biotechnological purposes.

The sequence of the monomer element used is: 5'-actagtCAGCCACCAAAGAGGACCCAGAATtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2, 4 and 8 copies of Box S were constructed and subjected to a transient expression assay as described above. The results were as follows:

30

	minus elicitor	Plus elicitor	Fold induction
1 x S	168	2058	12
2 x S	118	10781	91
4 x S	187	76904	441
8 x S	781	102211	130

These Box S constructs are novel and have high inducibility. Four copies of Box S appears to be the best with a very low background value (187) a high induced level (76904) and a very high fold induction (441 x, the highest of any of the constructs tested).

Example 2: Box D is capable of mediating elicitor inducible gene expression

Box D (TACAATTCAAACATTGTTCAAACAAGGAACC; SEQ ID NO: 11) is present in the parsley PR2 promoter and has never before been reported to be a *cis*-acting element. Box D was identified by DNaseI footprinting, by loss of function experiments in the context of the PR2 promoter and by gain-of-function experiments with monomers and multimers. Box D is a very strong elicitor-responsive element, a tetramer directing 10-fold elicitor-inducibility combined with a very high level of expression, whilst a dimer is less strong but gives 15-20-fold inducibility. This clearly shows Box D to be a promising element for biotechnological purposes.

The sequence of the element used is: 5'-actagtTACAATTCAAACATTGTTCAAACAAGGAACCtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2 and 4 copies of Box D were constructed and subjected to the transient expression assay described above. The results were shown below.

	minus elicitor	Plus elicitor	Fold induction
1 x D	346	4002	11
2 x D	1562	31331	20
4 x D	5519	61552	11

These Box D constructs are novel. Two copies of Box D may be the best with a moderate background value (1562), a high induced level (31331) and a good fold induction (20 x).

Example 3: Box U provides for elicitor inducible gene expression

Box U (ATGAAGTTGAAATTCAATAG; SEQ ID NO: 13) is present in the parsley PR2 promoter and has never before been reported to be a *cis*-acting element. Box U has been defined by DNaseI footprinting, by loss of function experiments in the context of the PR2 promoter and by gain-of-function experiments with monomers and multimers. Box U is a reasonably strong elicitor-responsive element, a tetramer directing 40-fold elicitor-inducibility.

The sequence of the element used is: 5'-actagtAGTTGAAATTCAATAA-GTTGAAATTCAATAtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters.

Constructs containing 2 copies of the above Box U sequence were constructed. The results of a transient expression assay are shown below. These therefore contain 4 copies of the Box U element (AGTTGAAATTCAATA; SEQ ID NO: 12). 1 or 2 copies of Box U are also active.

	minus elicitor	Plus elicitor	Fold induction
4 x U	100	3947	39

These Box U constructs are novel. Box U appears to be a moderately strong pathogen-responsive element with a good fold induction (about 40 x).

Example 4: Some W Boxes are capable of mediating elicitor inducible gene expression

The results obtained in accordance with the present invention clearly show that there are great differences between the different W Boxes that have been tested. Some are very strong (Box W2), some weak (Box W1), some are not active at all on their own (Box W3) and some are present as composite elements together with other *cis*-acting elements (Box N). The W Boxes also have differences outside of the core TGAC sequences:

Box W1: TTTGACC (SEQ ID NO: 1)

Box W2: TTCAGCC-N₇-TTGACC (SEQ ID NO: 3)

Box W3: TGAC-N₆-GTCA (SEQ ID NO: 5)

Box N: TTTGACC plus GCCACC (S Box) (SEQ ID NO: 8)

Box W_{Amy}: TTTGACC within TGAC-N₆-GTCA palindrome (SEQ ID NO: 6)

4.1 Box W1

Box W1 (CACACTTAATTTGACCGAGTAACATTCGCC; SEQ ID NO: 2) has previously been identified as a weak elicitor-responsive *cis*-element in the parsley PR1 promoters and a tetramer has been shown to be sufficient to direct elicitor-responsive expression in the parsley transient expression system (Rushton, 1996). Box W1 contains the W box sequence TTGACC and evidence suggests that these elements are bound by the WRKY class of transcription factors. As W boxes have also been found in the monocots Wild oat (Rushton, 1995) and maize (Raventos, 1995) and WRKY proteins have been found in an increasing number of plant species this suggests that the W box elements may be *cis*-acting elements in all plant species. Box W1 had never before been tested on its own for activity as a monomer or in combination with other elements and it was observed that a monomer directs elicitor-inducible expression (5-fold inducibility) and that Box W1 is also active in combination with other elements (see below).

The current results show Box W1 itself, however, to be a weak element. The sequence of the element used (the monomer) is: 5'-actagtCACACTTA-

ATTTGACCGAGTAACATTCGCCTctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. This construct is slightly different than the construct previously reported (Rushton, 1996) as the element is inserted into the SpeI/XbaI sites and not BamHI/BglII. Constructs containing 1, 2 and 4 copies of Box W1 were constructed and subjected to the transient expression assay. The results were as follows.

	minus elicitor	Plus elicitor	Fold induction
1 x W1	362	1495	4.1
2 x W1	299	2433	8.1
4 x W1	56	870	<15

The fold induction with 4 x W1 is similar to the previously reported values (Rushton, 1996). Comparison with values for other elements shows Box W1 to be a weak element.

4.2 Box W2

Box W2 (TTATTCAGCCATCAAAGTTGACCAATAAT; SEQ ID NO: 4) has previously been identified as a *cis*-acting element required for the elicitor responsive expression of parsley PR1 promoters in the transient expression system (Rushton, 1996). However, gain of function has been first demonstrated in accordance with the present invention. Box W2, like Box W1, contains a TTGACC element but the rest of the element is totally different and these other sequences play an important role, as a tetramer of Box W1 is a weak element with about 10-fold elicitor inducibility whereas Box W2 directs levels of expression up to 100 times higher than Box W1 with a 50-fold elicitor inducibility. It is shown for the first time that Box W2 alone, as a monomer or multimer, is a very strong elicitor-responsive element and that it is also active in combination with other elements.

The sequence of the element used (the monomer) is: 5'-actagtTTATTCAGCCATCAAAGTTGACCAATAATtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2,

4 and 8 copies of Box W2 were constructed and subjected to the transient expression assay. The following results were obtained.

	minus elicitor	Plus elicitor	Fold induction
1 x W2	770	8914	11
2 x W2	998	46651	46
4 x W2	2375	105685	44
8 x W2	7680	164454	21

W2 is the strongest elicitor-responsive *cis*-acting element that has been so far tested, eight copies of W2 giving GUS values of approximately 164,000.

4.3 Box N

Box N comes from the potato *gst1* gene (TTCTAGCCACCAGATTTGACCAAAC; SEQ ID NO: 9) and has never previously been defined. It contains both an S Box sequence (AGCCACCAGA) and a W Box sequence (TTGACC) within just 25 base pairs and as such represents a novel *cis*-element composed of two types of elicitor response element within a very small stretch of DNA. A tetramer of Box N gives at least 75-fold elicitor inducibility. This observation suggests three important conclusions; firstly that Box N may be extremely useful for biotechnological applications, secondly that the core Box S sequence is AGCCACCANA (SEQ ID NO: 14) and thirdly that Boxes S and W may represent a common theme in plant promoters that respond to pathogens as these elements are present in both parsley and potato. Box N alone is a strong elicitor-responsive element and extremely interesting, as it consists of an S Box (GCCACC) followed by a W Box (TTTGACC). The sequence of the element used (the monomer) is: 5'-actagtTTCTAGCCACCAGATTTGACCAAACtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. A construct with four copies of Box N was tested in transient expression assay. The results were as follows.

35

	minus elicitor	Plus elicitor	Fold induction
4 x N	1085	92980	85

Box N is a strong element and shows a very high fold inducibility. This novel combination and spacing of W and S Box elements may prove to be very useful for biotechnological purposes.

4.4 Box W_{Amy}

Box W_{Amy} comes from the wild oat α -Amy2/A and wheat α -Amy2/54 genes where it has previously been published under the name Box 2 or O2S (see Rushton, Plant Mol. Biol. 29 (1995), 691-702). It is a *cis*-acting element required for the transcriptional activation of these genes during germination but has never previously been linked to a role in pathogenesis. Box W_{Amy} consists of two W Box elements: a hexamer 5'-TTGACC-3' embedded in a palindromic 5'-TGAC-N₆-GTCA-3'. As it contains both types of sequences together it constitutes a new type of W Box and may be a "super W Box".

The sequence of the element used (the monomer) is: 5'-actagtGGATTGACTTGACCGTCATCGGCTtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. A construct containing 7 copies of Box W_{Amy} was constructed and to the transient expression assay. The result is shown below.

	minus elicitor	Plus elicitor	Fold induction
7 x W _{Amy}	168	43867	260

W_{Amy} is a strong elicitor-responsive *cis*-acting element and has the highest fold induction of any W Box that has been so far tested. This element could therefore be a particularly effective W Box and could aid the designing of synthetic W Boxes that are even more effective

Example 5: Synthetic promoters consisting of combinations of the above-described elements

Synthetic promoters composed of combinations of the above elicitor-responsive elements have never before been constructed or tested. All elements (Boxes W1, W2, S, U, D, N and W_{Amy}) are active in combination with each other; monomer, dimer and tetramer constructs being active. The furthest downstream element (nearest to the TATA Box) has the strongest effect on the synthetic promoter with further upstream elements having a much lesser effect. However the combination of two or more different types of *cis*-element may have a much more profound effect on expression *in planta*. In addition the insertion of a spacer region composed of anything between 100 base pairs and 1,000 base pairs appears to increase the contribution of the more upstream *cis*-elements. All of these synthetic promoters are good candidate promoters that may be rapidly and locally responsive to pathogen attack but also show negligible activity in uninfected tissues. These promoters may therefore allow the engineering of defense reactions that are closely related to natural defense mechanisms without appreciable activity in non-infected cells of the plant.

A large number of combinations have been tested. The results for some of these are detailed below. All of these combinations are novel and these constructs represent true synthetic promoters. The elements are inserted into the *SpeI*/*XbaI* sites, as with all of the constructs, and read from the 5' end to the 3' end i.e. 4 x W2/ 4 x S is:

SpeI - W2 - W2 - W2 - W2 - S - S - S - S - *XbaI*

Generally, the elements nearest to the TATA Box (i.e. at the 3' end) have the greatest effect on both level of expression and fold induction. The effect of the upstream elements is often minimal and there is also an inhibitory effect probably due to steric hindrance when different elements are put close together; compare 4 x S/ 4 x W2 with (2 x S/ 2 x W2) x 2. The insertion of spacer regions between elements is therefore recommended to alleviate problems due to steric hindrance. The results of the transient expression assays are shown below.

	minus elicitor	Plus elicitor	Fold induction
1 x S/ 1 x W2	1732	85126	49
2 x S/ 2 x W2	1529	95872	62
4 x S/ 4 x W2	2654	64105	24
(2 x S/ 2 x W2) x 2	483	9832	20
4 x W2/ 4 x S	2753	205826	74
1 x W2/ 1 x S	146	2690	18
2 x S/ 2 x D	191	15541	81
4 x S/ 4 x D	9775	100265	10
1 x D/ 1 x S	32	1246	38
4 x D/ 4 x S	6795	204115	30
2 x W2/ 2 x D	1762	32462	18
4 x W2/ 4 x D	22042	92875	4.2
4 x D/ 4 x W2	18857	276456	14
1 x D/ 1 x W2	295	4369	14

Adding more copies of an element in a composite construct often increases the absolute level of expression (e.g. 2 x W2/ 2 x D and 4 x W2/ 4 x D) but often lowers the fold induction. In some cases even the absolute level of expression decreases (e.g. 2 x S/ 2 x W2 and 4 x S/ 4 x W2) and a comparison with (2 x S/ 2 x W2) x 2 suggests that this is due to steric hindrance as the number of copies of the elements is the same, it is just the order that is changed.

Example 6: Box E17 is capable of mediating elicitor induced gene expression

Box E17 (TCAATATGTCAATGGTCAACATTCAAC; SEQ ID NO: 15) was isolated from the promoter of the parsley Eli17 gene which is known to react to elicitor-treatment with transcript accumulation (Somssich, Plant Mol. Biol. 12 (1989), 227-234). Recently it has been shown that the Eli17 gene reacts very rapidly and transiently to elicitor-treatment and pathogen infection. This has never been previously described.

The sequence of the monomer element used is: 5'-actagtTCAATA-TGTCAATGGTCAACATTCAACtctaga-3' with the element in upper case letters and

the *SpeI/XbaI* ends in lower case letters. Constructs containing 1 and 2 copies of Box E17 as well as a monomeric reverse complement of Box E17 were constructed (Figure 4, constructs B109, A109, and 18S102, respectively) and subjected to a transient expression assay as described above. As shown in Figure 4, the monomer has 5-fold inducibility and the dimer 50-fold. In comparison to the other *cis*-elements of the present invention moderate induction was achieved by Box E17. Likewise, a tetramer of Box E17 was subjected to transient assays (data not shown), which resulted in 5- to 20-fold induction following elicitor-treatment. However, this result cannot be compared to the induction values of the Box E17 constructs mentioned above because of diminished quality of the parsley protoplasts used. Presumably, the Box E17 tetramer mediates at least an induction as high-fold as the respective dimer.

Similar to *cis*-elements of Example 4, Box E17 contains two copies of the W-Box core motif TGAC, in reverse orientation (GTCA) as tandem repeat separated by a 3 bp spacer. The importance of this core motif can be inferred from preliminary mutagenesis experiments (Figure 4, constructs C109, 17S102, and 15S102). A 1 bp deletion within the W-Box motif resulted in complete loss of function in contrast to deletions at two different sites having no effect to inducibility. In order to further narrow down the minimal structure capable of mediating elicitor-responsiveness dissected Box E17 elements were tested in transient expression assays as described above. The initial Box E17 (SEQ ID NO: 15) was deleted from the 5'-end by 6 bp, from the 3'-end by 7 bp and from both ends by 6 and 7 bp, respectively. Each of these oligonucleotides were ligated into the *Bam*HI site of the MS23 vector, which was before cut with *Bam*HI restriction enzyme and the overhangs blunted, giving rise to the promoter constructs H149, B175, C175 and D175 (Figure 5). The promoter constructs showed remarkable differences regarding their elicitor-responsiveness. C175 and D175 having the 3'-end truncated, displayed no significant induction upon elicitor treatment. On the other hand, the 5'-truncated B175 gave values which were similar to those of the 27 bp Box E17 element. Thus, also the 21 bp-element B175 (SEQ ID NO: 16) is a functional *cis*-element in the sense of the present invention.

Furthermore, Box E17 is not only sufficient but also necessary to lend the *Eli*17 promoter, or at least a 445 bp long functional part thereof which comprises said element naturally, its elicitor inducibility. This could be shown in transient expression

assays which were performed with an MS23-construct containing the 445 bp stretch having the 27 bp-element removed. The resulting complete loss of elicitor-dependent inducibility (see Figure 5) indicates the crucial role of Box E17 for elicitor- and pathogenesis-related gene regulation in its natural environment and further supports its applicability for conferring inducibility upon elicitation or pathogenesis to a chimeric promoter according to the present invention.

Example 7: Chimeric promoters with varying distances of the Box E17 element to the minimal promoter are inducible.

In order to elucidate the optimal position of the Box E17 element within the chimeric promoter several constructs with varying distances to the 35S minimal promoter were tested (Figures 6 and 7). For this purpose Box E17 was inserted into different restriction sites of the ms23 polylinker. After digesting the vector and filling in the overhangs, the *cis*-element was blunt ligated into the respective site as a monomer or as a dimer. The transient assays were conducted as described above. The results (Figure 7) indicate an optimal distance of Box E17 to the 5' end of the minimal promoter of 40 to 60 bp (corresponding to the restriction sites BamHI, ClaI, EcoRI). Still good induction was observed for the SapI site in 131 bp distance whereas considerably weaker response was obtained when Box E17 was inserted into the Sall site which is 5 bp upstream of the minimal promoter.

Example 8: Transgenic plants carrying chimeric promoters

Transformants were tested for the response of the synthetic promoters to pathogens. Cultures of the bacterium *Pseudomonas* (strains *Rpt2* or *Rpm1*) were grown in King's-B Medium containing 30 µg/ml kanamycin and 100µg/ml rifampicin. The bacteria were resuspended in 10mM MgCl₂ at an OD₆₀₀ of 0.2 and infiltrated into leaves via a syringe. Controls were performed using 10mM MgCl₂ alone. After 6 hours the leaves were removed from the plants and stained for GUS activity using X-Gluc. The expression pattern observed in the transgenic plants containing the GUS marker gene under the control of the chimeric promoter of the invention revealed expression in tissue infected by *Pseudomonas syringae* and in some cases also local expression in wounded tissues.

With regard to Box E17 a chimeric promoter comprising the dimer of this element (A109, Figure 4) and the 35S minimal promoter was used for transformation of *Arabidopsis* plants. Two to three weeks old seedlings and old leaves of the transformants were infiltrated with a 10 μ M aqueous solution of the bacterial elicitor Flagellin 22 via a syringe (Felix, Plant Journal 18 (1999) 262-276; Gómez-Gómez, Plant Journal 18 (1999) 277-284) which led to clear GUS activation. High induction was also observed after infection by a fungal (*Peronospora parasitica*) and a bacterial pathogen (*Pseudomonas syringae*).

Peronospora infections were carried out according to Dangl et al. (Genetic definition of loci involved in Arabidopsis-pathogen interactions. In: Methods in Arabidopsis Research (Koncz, Chua and Schell, eds.). Singapore: World Scientific Publishing Co. (1992), 393-418) or Koch (Plant Cell 2 (1990), 437-446).

On the other hand, mechanical stress induced for example by wounding did not activate the chimeric promoter. And surprisingly, no or only mere expression and activation of the reporter gene was observed in root which is the organ where the Eli17 gene is predominantly expressed in parsley. Thus, organ specificity appears not to be mediated by Box E17.

Furthermore, expression studies were performed the results of which are summarized in Figure 8. Seven different tetramers of *cis*-elements were assayed for their background expression in aerial parts and roots, respectively, and for their inducibility after wounding, senescence, incompatible and compatible *Peronospora* infection. Some important conclusions can be drawn from these experiments:

All of these chimeric promoters that are inducible by incompatible strains of *Peronospora parasitica* are also inducible by compatible strains. This is an important observation regarding the present invention as it shows that these constructs could be inducible by all potential pathogens and not just those for which there is already a functional defense system in operation in the plant.

Although many constructs show induced expression around infection sites, the expression characteristics are different with, for example, some W Boxes (e.g. W2) being expressed in an area around the infection site whereas others are expressed within the infection site itself. This is an unexpected finding as it shows that within a

class of *cis*-acting elements (W Boxes or GCC/S Boxes) differences in sequence outside of the core sequence lead to differences in functionality.

All of the *cis*-acting elements of the present invention show inducible expression in a heterologous plant (*Arabidopsis*). As these elements come from parsley, potato and wheat this clearly shows that these elements could be functional in all plants. This general functionality of such elements is an important new observation.

CLAIMS

1. A chimeric promoter capable of mediating local gene expression in plants upon pathogen infection comprising
 - (i) at least one *cis*-acting element sufficient to direct elicitor-specific expression comprising the nucleotide sequence of any one of SEQ ID NOS: 3 to 16, and
 - (ii) a minimal promoter.
2. The chimeric promoter of claim 1, further comprising a *cis*-acting element having the nucleotide sequence of SEQ ID NO: 1 or 2.
3. The chimeric promoter of claim 1 or 2, wherein said synthetic plant promoter comprises homo- and/or hetero-multimeric forms of said *cis*-acting element(s).
4. The chimeric promoter of any one of claims 1 to 3, wherein said multimeric form is a dimer or tetramer.
5. The chimeric promoter of any one of claims 1 to 4, wherein the minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, or hcbt2 promoter.
6. The chimeric promoter of any one of claims 1 to 5, wherein the distance between said *cis*-acting element and said minimal promoter is 12 to 300 base pairs, more preferably 25 to 70 base pairs, and most preferably 40 to 60 base pairs.
7. The chimeric promoter of any one of claims 1 to 6, wherein a spacer region composed of 4 to 10 base pairs separates at least two of said *cis*-acting elements.
8. The chimeric promoter of any one of claims 3 to 7, wherein at least two of said multimeric forms are separated by a spacer of between about 50 to 1000 base pairs.

9. The chimeric promoter of any one of claims 1 to 8, wherein the induction of gene expression upon elicitor treatment or pathogen infection is at least 15-fold.
10. A recombinant gene comprising the chimeric promoter of any one of claims 1 to 9.
11. The recombinant gene of claim 10, wherein the chimeric promoter is operatively linked to a heterologous DNA sequence.
12. The recombinant gene of claim 10 or 11, wherein at least one of said *cis*-acting elements is located in the 5'- or 3-untranslated region or in an intron of the recombinant gene.
13. The recombinant gene of claim 11 or 12, wherein said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme, transcription factor, protease, nuclease, lipase, or polymerase.
14. A vector comprising the chimeric promoter of any one of claims 1 to 9 or the recombinant gene of any one of claims 10 to 13.
15. A method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a chimeric promoter of any one of claims 1 to 9, a recombinant gene of any one of claims 10 to 13 or the vector of claim 14 into the genome of said plant, plant cell or plant tissue.
16. Plant cells comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13 or the vector of claim 14 or obtainable by the method of claim 15.
17. A transgenic plant or plant tissue comprising plant cells of claim 16.

18. The transgenic plant of claim 17, which upon the presence of the chimeric promoter or the recombinant gene attained resistance or improved resistance against a pathogen the corresponding wild-type plant was susceptible to.
19. Harvestable parts of a transgenic plant of claim 17 or 18 comprising plant cells of claim 16.
20. Propagation material of a transgenic plant of claim 17 or 18 comprising plant cells of claim 16.
21. A *cis*-acting element as defined in claim 1 or a multimeric form(s) of any one of those as defined in claim 3 or 4.
22. A method for the identification of an activator or inhibitor of genes specifically expressed in plants upon pathogen infection comprising the steps of:
 - (a) providing a plant, plant cell, or plant tissue comprising a recombinant DNA molecule comprising a readout system operatively linked to the chimeric promoter of any one of claims 1 to 9;
 - (b) culturing said plant cell or tissue or maintaining said plant in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said readout system;
 - (c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or plant tissue.
23. The method of claim 22 further comprising the step of
 - (d) subdividing the samples identified in step (c) and repeating steps (a) to (c) one or more times.
24. The method of claim 22 or 23 further comprising the step of
 - (e) identifying and/or isolating from the identified sample the compound responsible for said suppression or activation and/or enhancement of

expression of said readout system in said plant, plant cell, or plant tissue.

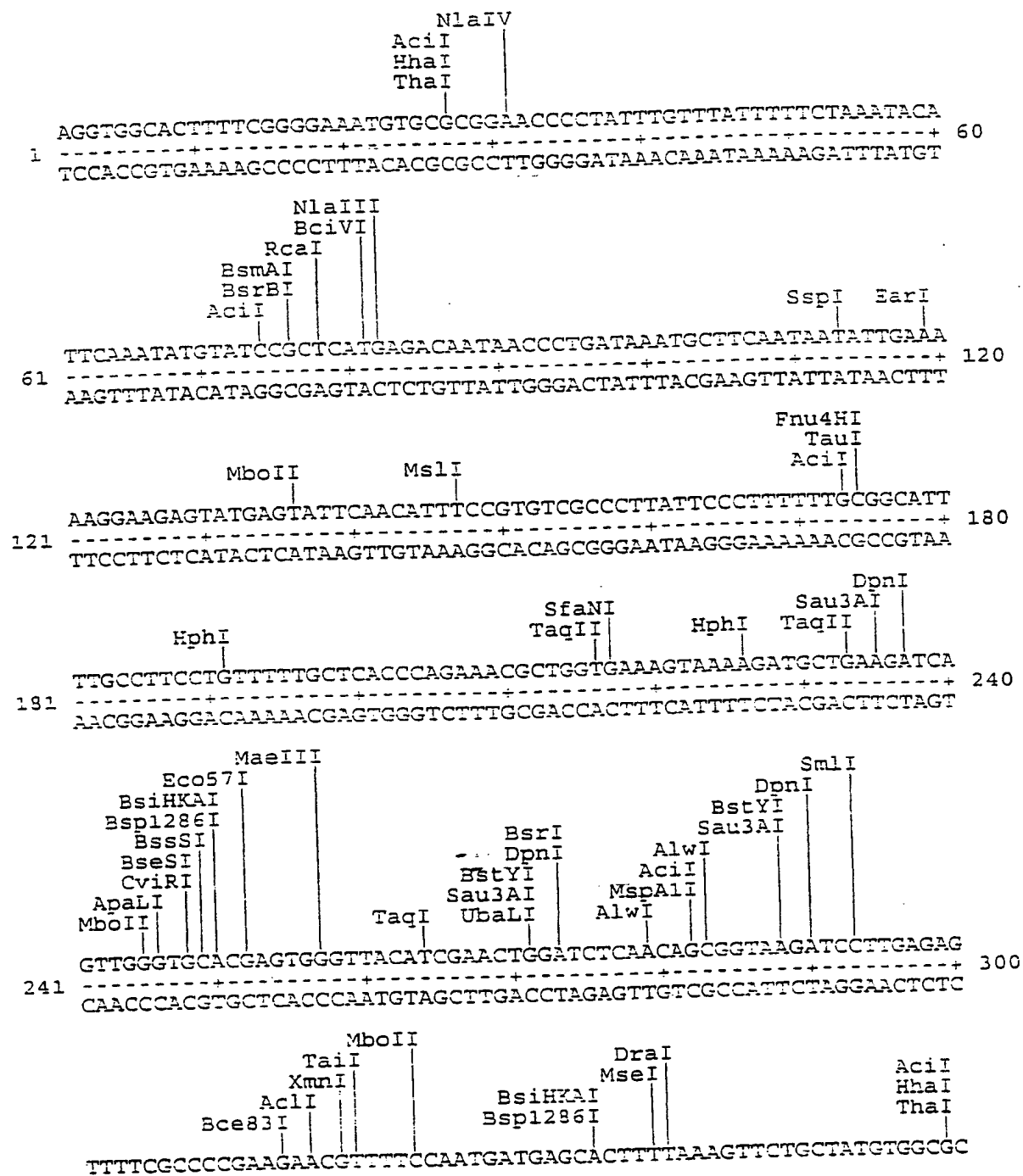
25. The method of any one of claims 22 to 24, wherein
 - (a) said recombinant DNA molecule is a recombinant gene of any one of claims 10 to 13 or a vector of claim 14;
 - (b) said plant cell is a plant cell of claim 16;
 - (c) said plant tissue is a plant tissue of claim 17, or
 - (d) said plant is a plant of claim 17 or 18.
26. A method for preparing a plant elicitor comprising the steps of the method of any one of claims 22 to 25 and formulating the compound obtained or identified in step (c) or (e) in a form suitable for the application in agriculture or plant cell and tissue culture.
27. A compound obtained or identified by the method of any one of claims 22 to 26 which is an activator or inhibitor of gene expression and/or function in plants.
28. An antibody specifically recognizing the compound of claim 27 or the *cis*-acting element of claim 21.
29. A diagnostic composition comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the compound of claim 27 or the antibody of claim 28, and optionally suitable means for detection.
30. A kit comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the compound of claim 27 or the antibody of claim 28.
31. A plant protection composition comprising the compound of claim 27.

32. Use of a *cis*-acting element sufficient to direct elicitor-specific expression, a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the *cis*-acting element of claim 21 and/or the compound of claim 27 for the production of pathogen resistant plants.
33. Use of a *cis*-acting element sufficient to direct elicitor-specific expression, the chimeric promoter of any one of claims 1 to 9, a recombinant gene of any one of claims 10 to 13, a vector of claim 14, the plant cell of claim 16, the plant tissue of claim 17, or the plant of claim 17 or 18 for identifying and/or producing compounds capable of conferring induced resistance to a pathogen in a plant.
34. A method of rendering a gene responsive to pathogens comprising inserting at least one *cis*-acting element sufficient to direct elicitor-specific expression into the promoter of said gene.
35. A method for preparing a promoter capable of mediating local gene expression in plants upon pathogen infection comprising operably linking a *cis*-acting element sufficient to direct elicitor-specific expression to a transcription initiation sequence of a promoter.
36. The method of claim 34 or 35, wherein said *cis*-acting element is a *cis*-acting element as defined in claim 1 or 2 or a multimeric form thereof as defined in any one of claims 3 to 8.
37. The method of any one of claims 34 to 36, further comprising deleting non-specific *cis*-acting elements in the promoter.
38. The promoter obtainable by the method of any one of claims 34 to 37.
39. Use of the compound of claim 27 as plant protective agent or herbicide.

1/21

Fig. 1

Restriction map of plasmid ms23

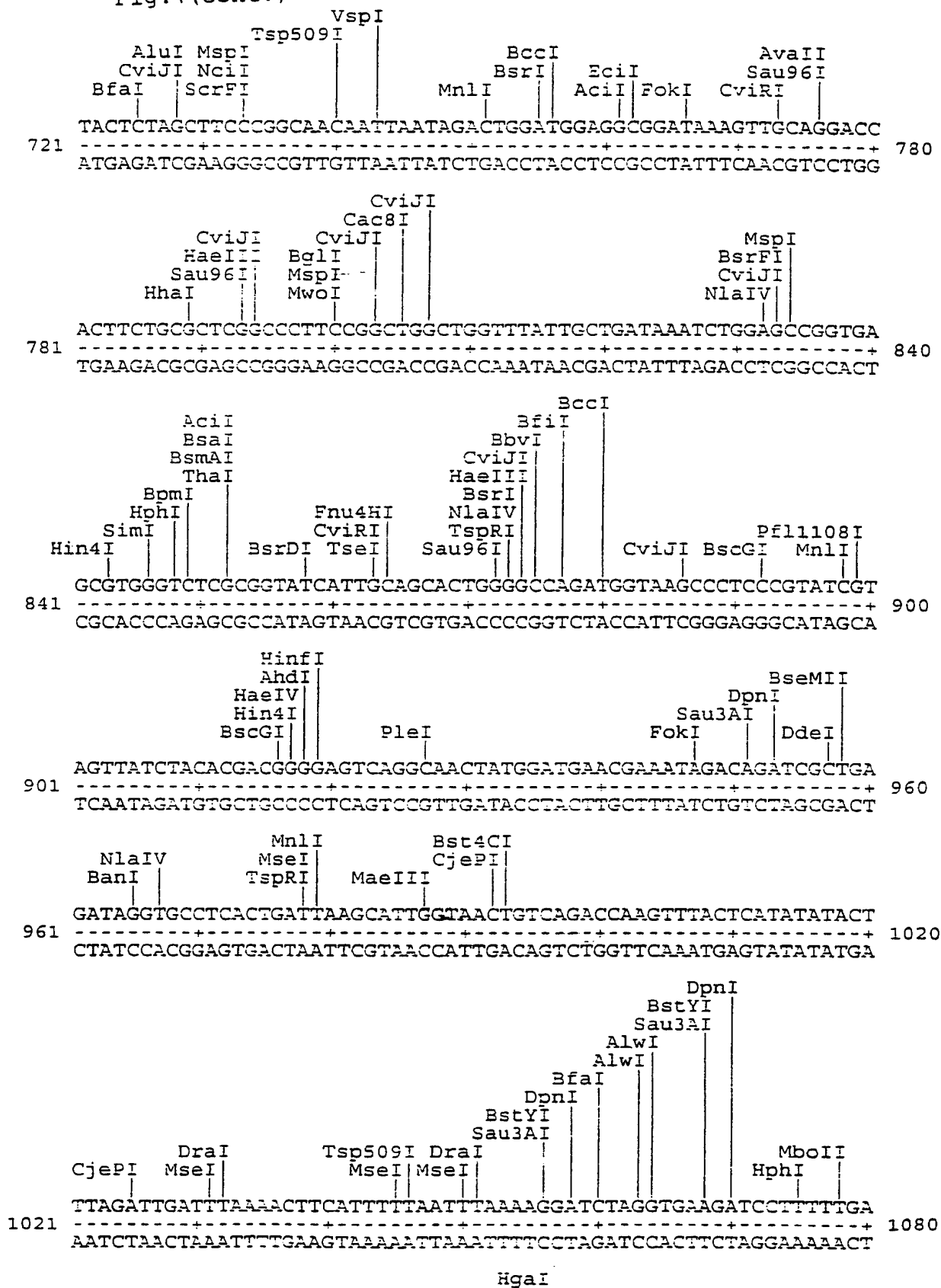


2/21

MseI

3/21

Fig.1(cont.)



4/21

Fig.1 (cont.)

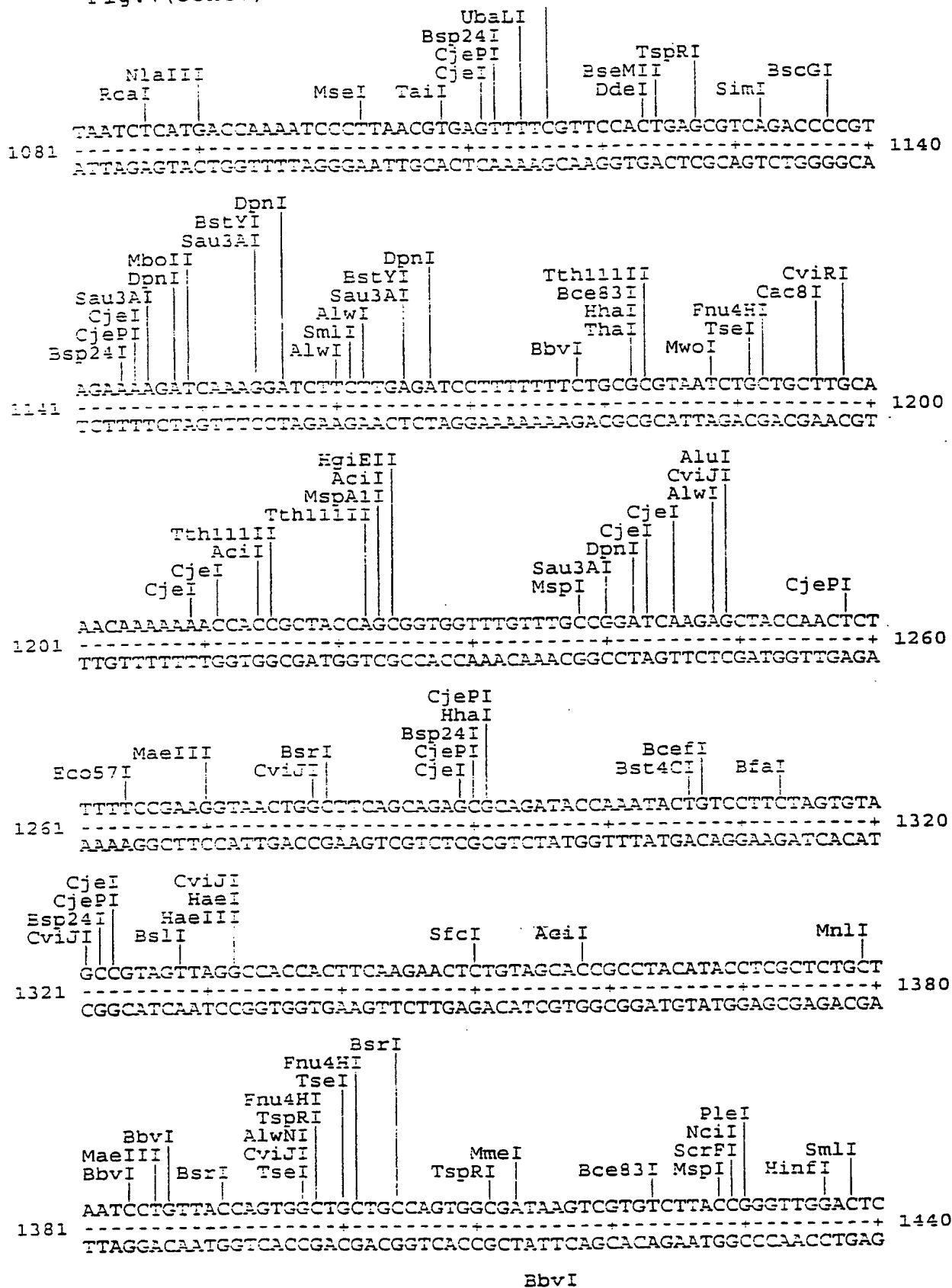
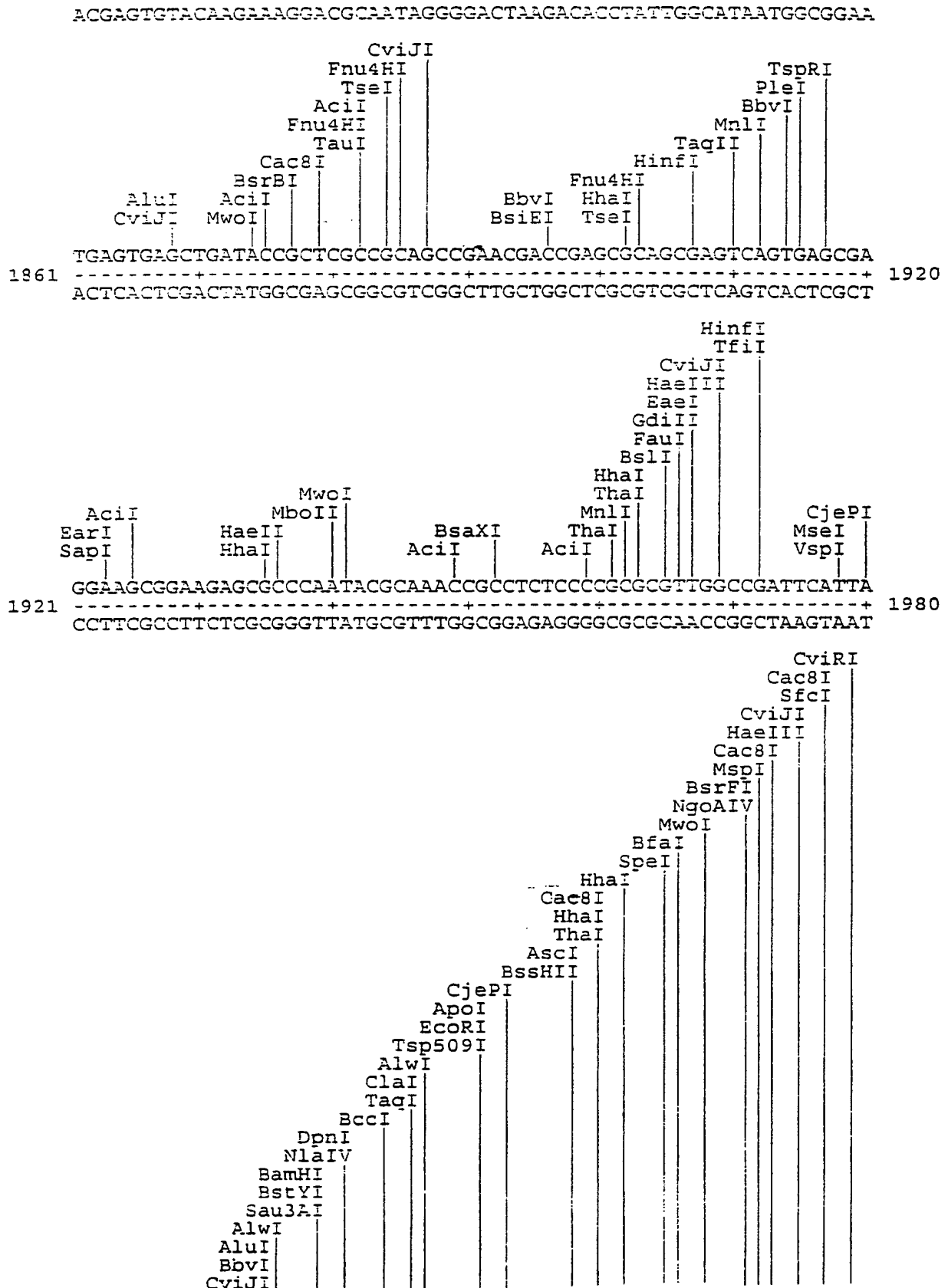


Fig. 1 (cont.)

SUBSTITUTE SHEET (RULE 26)

Fig.1 (cont.)

6/21



7/21



8/21

SUBSTITUTE SHEET (RULE 26)

Fig.1 (cont.)



Fig.1 (cont.)

10/21

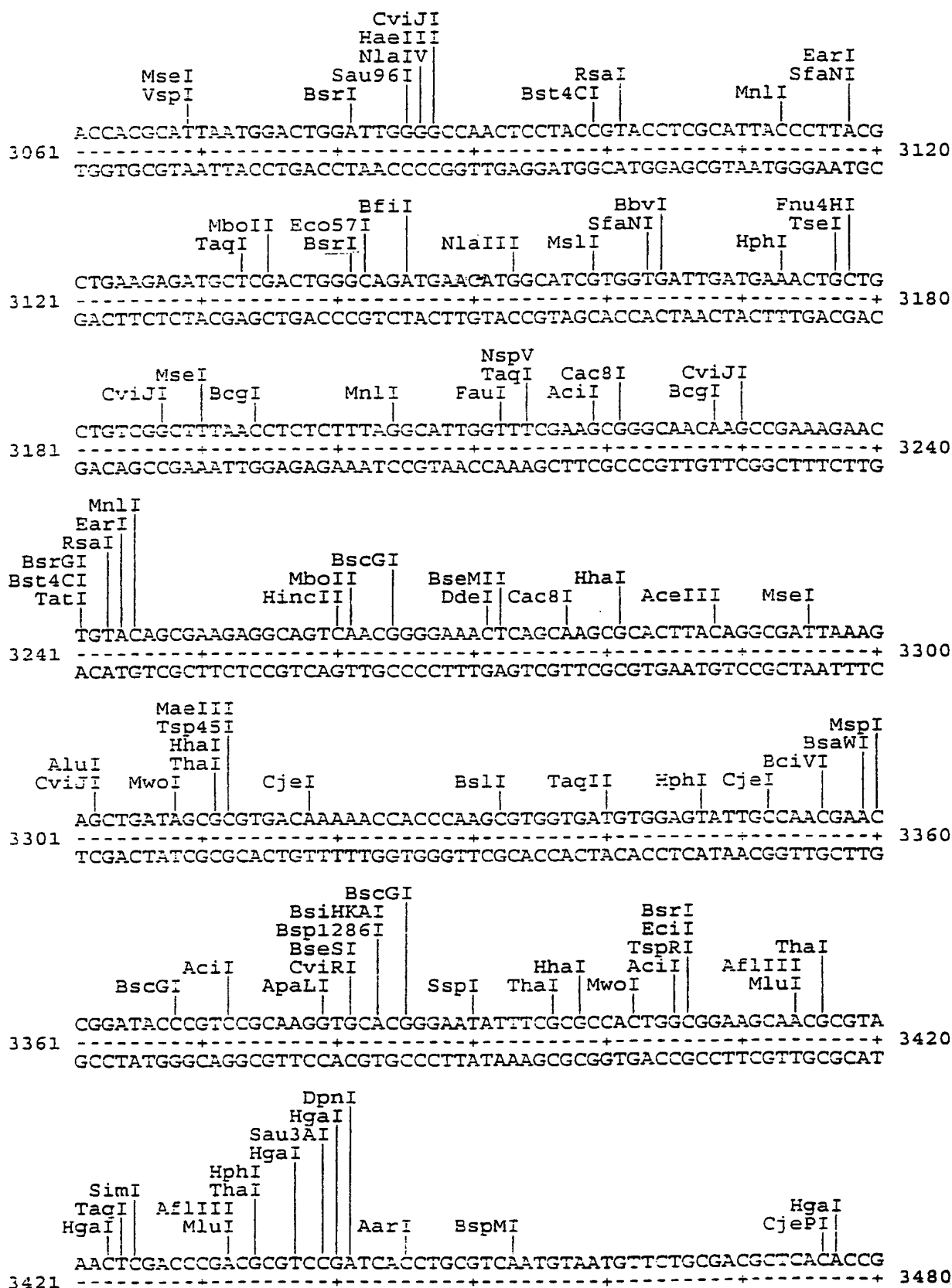


Fig.1(cont.)

11/21

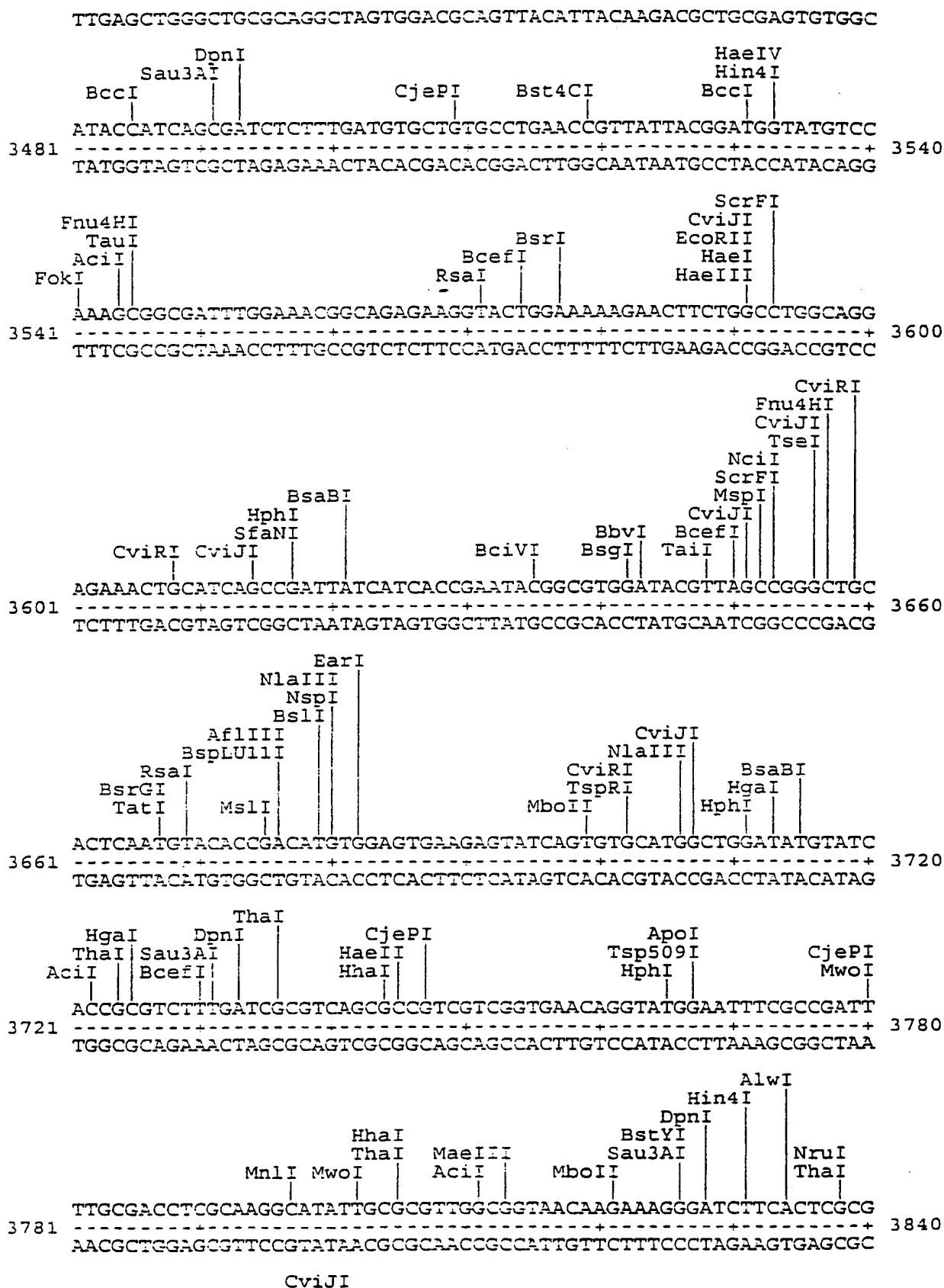
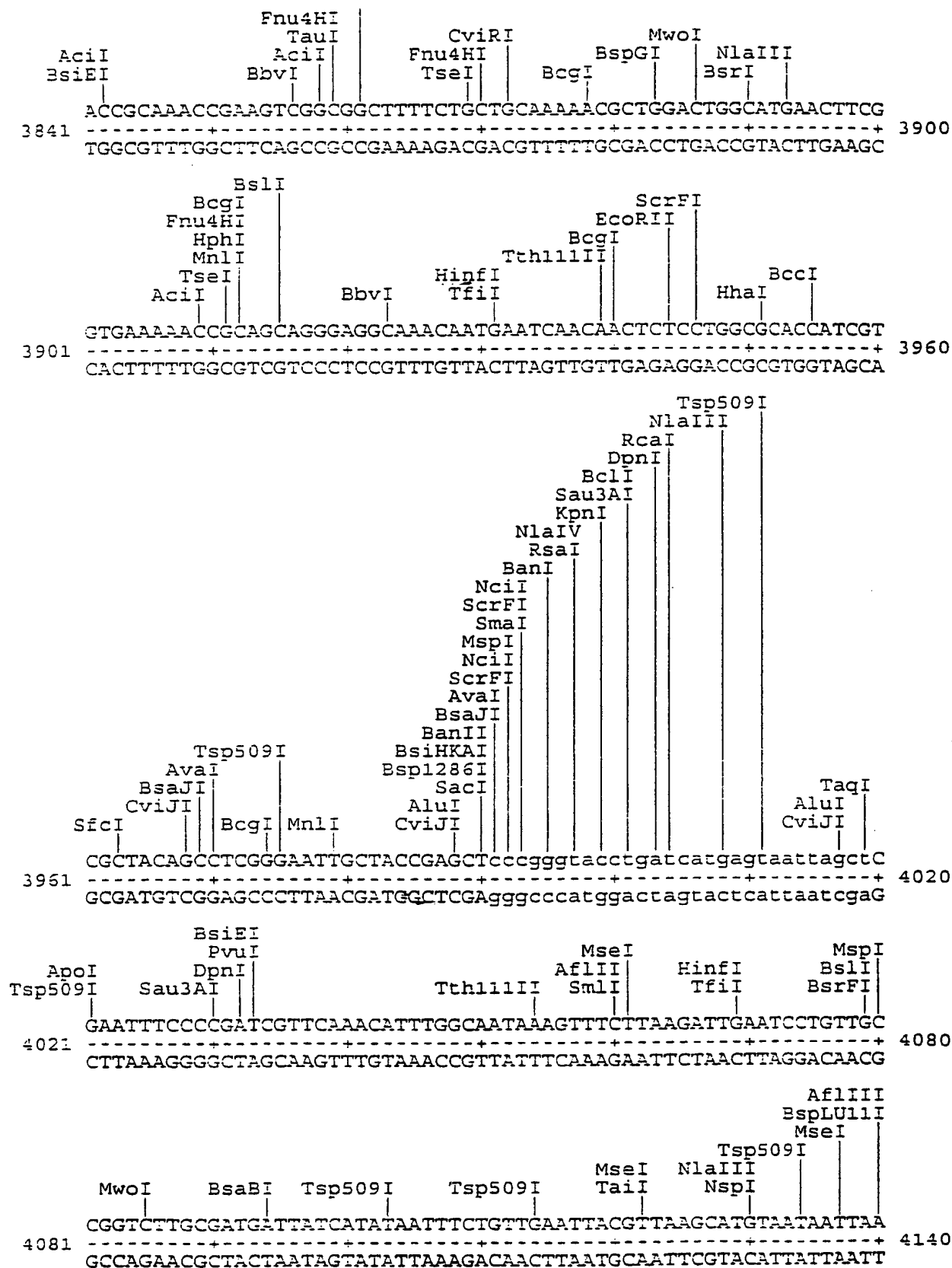
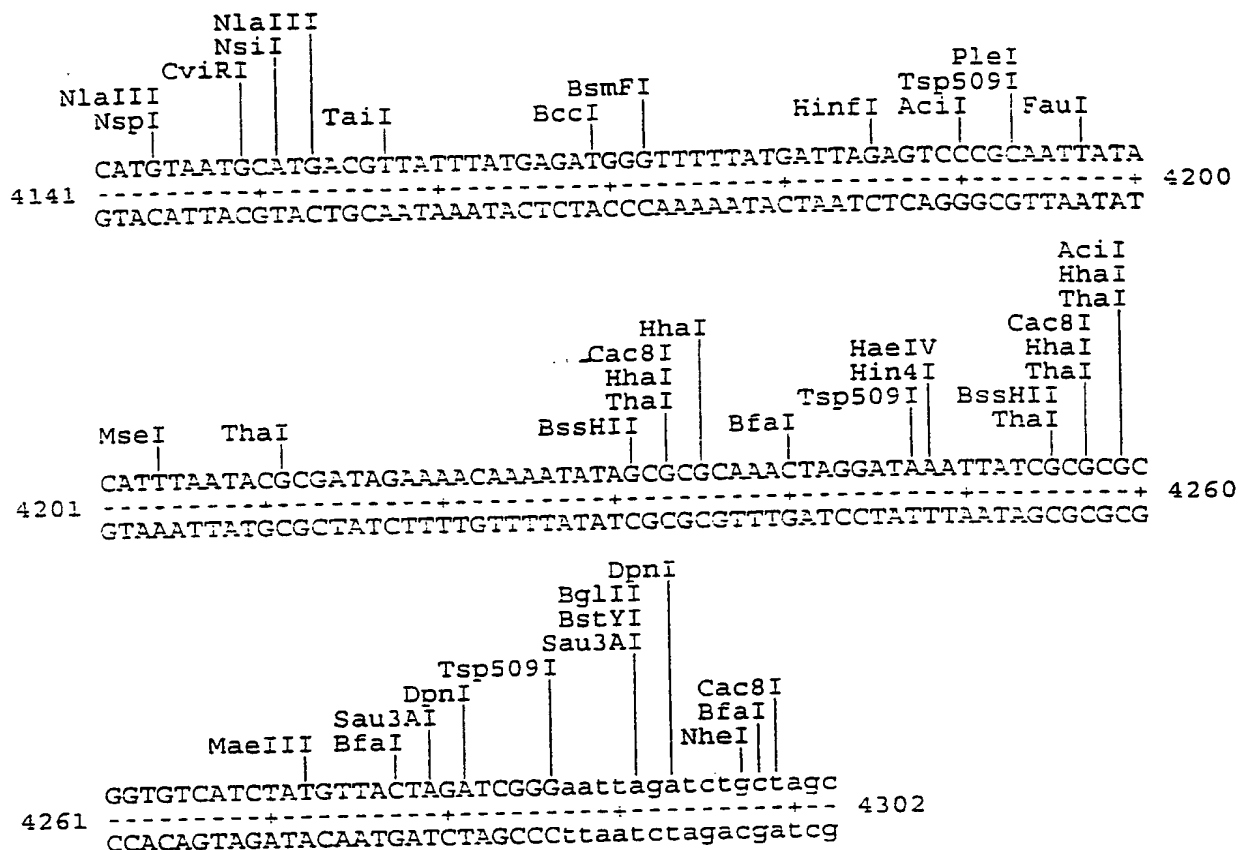


Fig.1 (cont.)

12/21



13/21



AarI	AccI	AceIII	AcII	AcII	AflII	AflIII	AhdI
AluI	AlwI	AlwNI	ApaII	ApoI	AscI	AvaI	AvaII
BamHI	BanI	BanII	BbvI	BccI	Bce83I	BceFI	BcgI
BciVI	BclI	BfaI	BfiI	BglI	BglII	BpmI	BsaI
BsaAI	BsaBI	BsaHI	BsaJI	BsaWI	BsaXI	BscGI	BseMI
BseSI	BsgI	BsiEI	BsiHKAII	BslI	BsmI	BsmAI	BsmFI
Bsp24I	Bsp1286I	BspGI	BspLU11I	BspMI	BsrI	BsrBI	BsrDI
BsrFI	BsrGI	BsshII	BssSI	Bst4CI	BstDSI	BstyI	BtsI
Cac8I	CjeI	CjePI	Clal	CviJI	CviRI	DdeI	DpnI
DraI	DrdI	EaeI	EaeI	EciI	Eco57I	EcoRI	EcoRII
EcoRV	FauI	Fnu4HI	FokI	FspI	GdiII	HaeI	HaeII
HaeIII	HaeIV	HgaI	HgiEII	HhaI	Hin4I	HincII	HindIII
HinfI	HphI	KpnI	MaeIII	MboII	MluI	MmeI	MnlI
MscI	MseI	MslI	MspI	MspAII	MunI	MwoI	NciI
NcoI	NgoAIV	NheI	NlaIII	NlaIV	NruI	NsiI	NspI
NspV	Pfl1108I	PleI	PshAI	PstI	PvuI	RcaI	RleAI
RsaI	SacI	SalI	SapI	Sau96I	Sau3AI	ScaI	ScrFI
SfaNI	SfcI	SimI	SmaI	SmlI	SnaBI	SpeI	SspI
StyI	TaiI	TaqI	TaqII	TatI	TauI	TfiI	ThaI
TseI	Tsp45I	Tsp509I	TspRI	Tth111II	UbaLI	VspI	XbaI
XhoI	XmnI						

AatII	AloI	ApaI	AvrII	BaeI	BbsI	BbvCI	BmgI
BplI	Bpu10I	Bpu1102I	BsbI	BseRI	BsmBI	BspEI	BstAPI
BstEII	BstXI	BstZ17I	Bsu36I	DraIII	DrdII	EagI	Eco47III
EcoNI	EcoO109I	FseI	HpaI	NarI	NdeI	NotI	PacI
PflMI	PinAI	PmeI	PmlI	Psp5II	PvuII	RsrII	SacII
SanDI	SbfI	SexAI	SfiI	Sgfi	SgrAI	SphI	SrfI
Sse8647I	StuI	SunI	SwaI	Tth111I	XcmI		

14/21

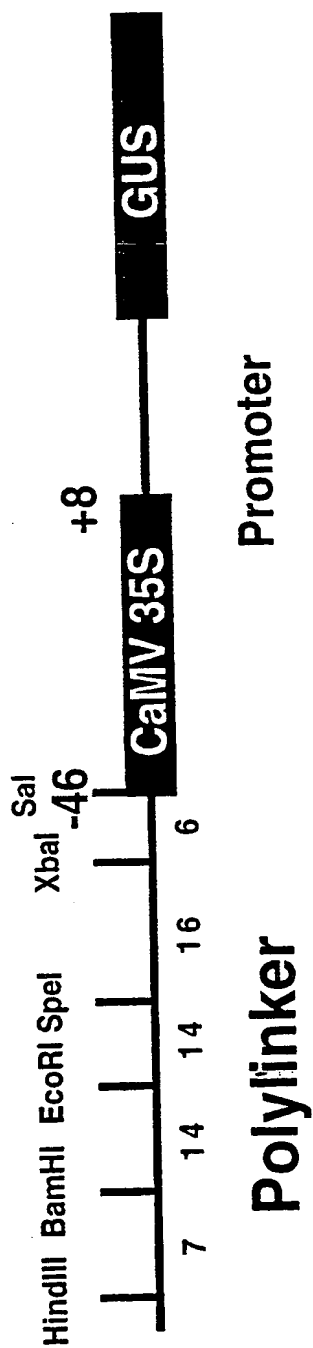
MS23

Fig. 2

pGPTV-GUS-kan

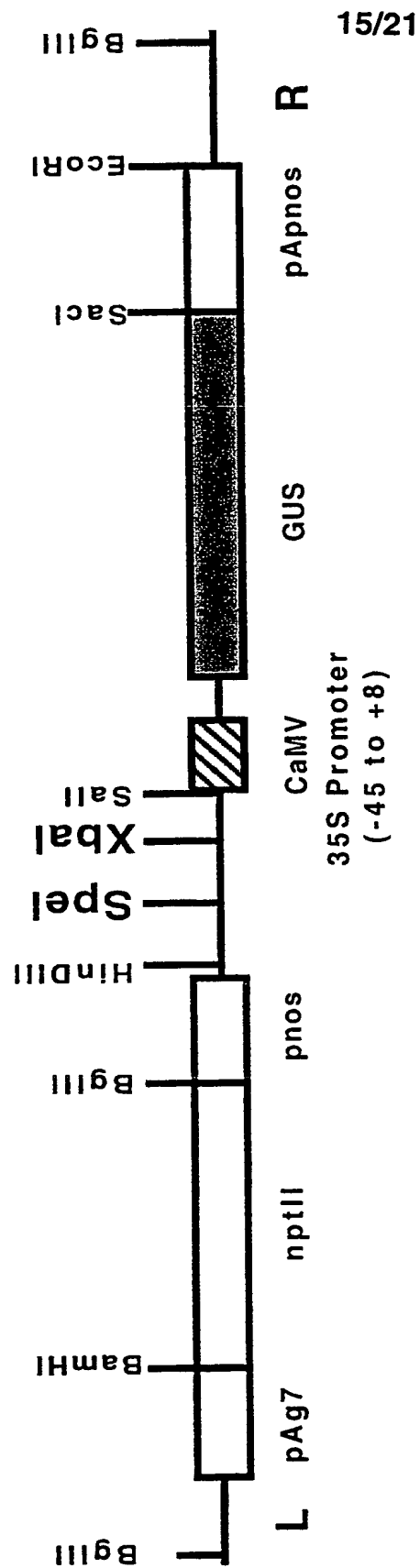


Fig. 3

16/21

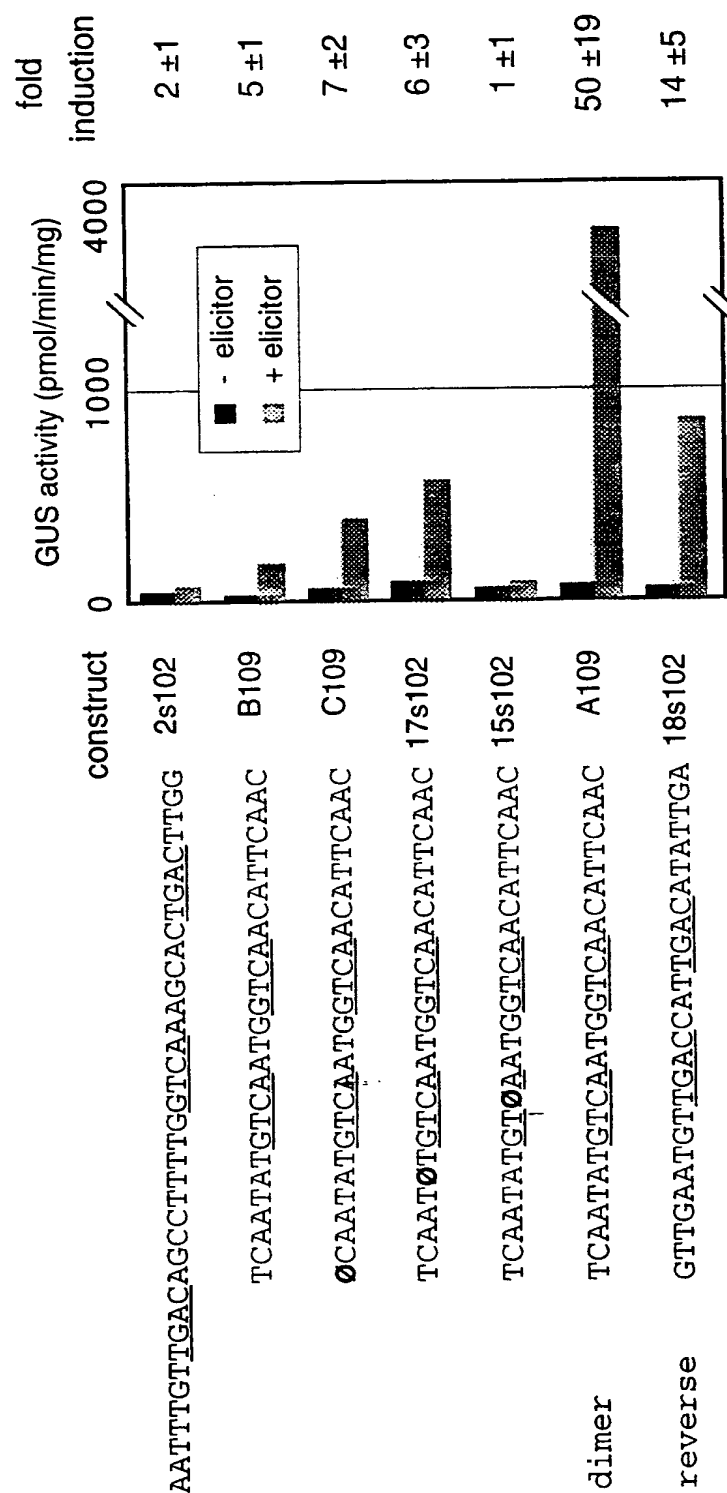


Fig. 4

17/21

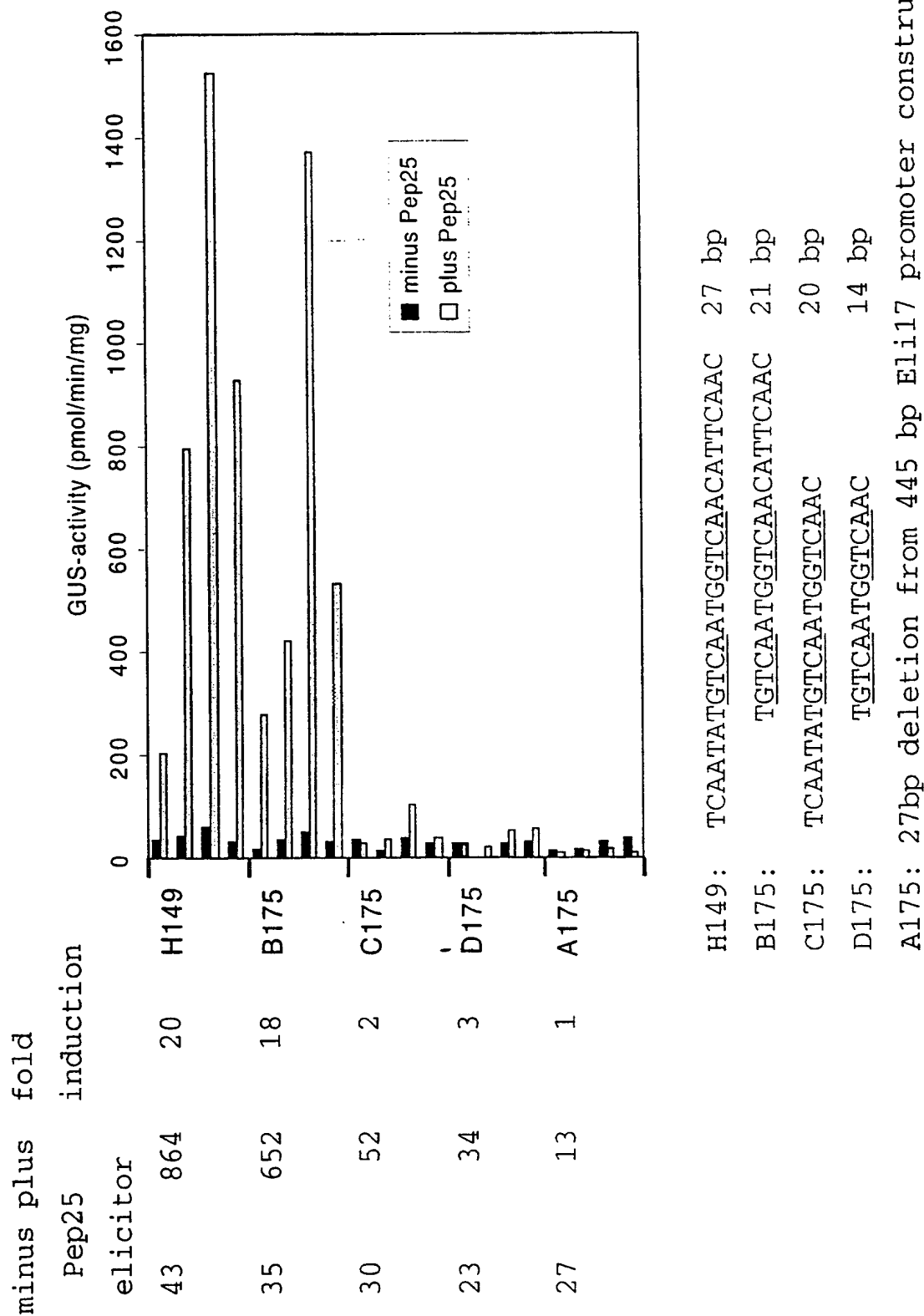


Fig. 5

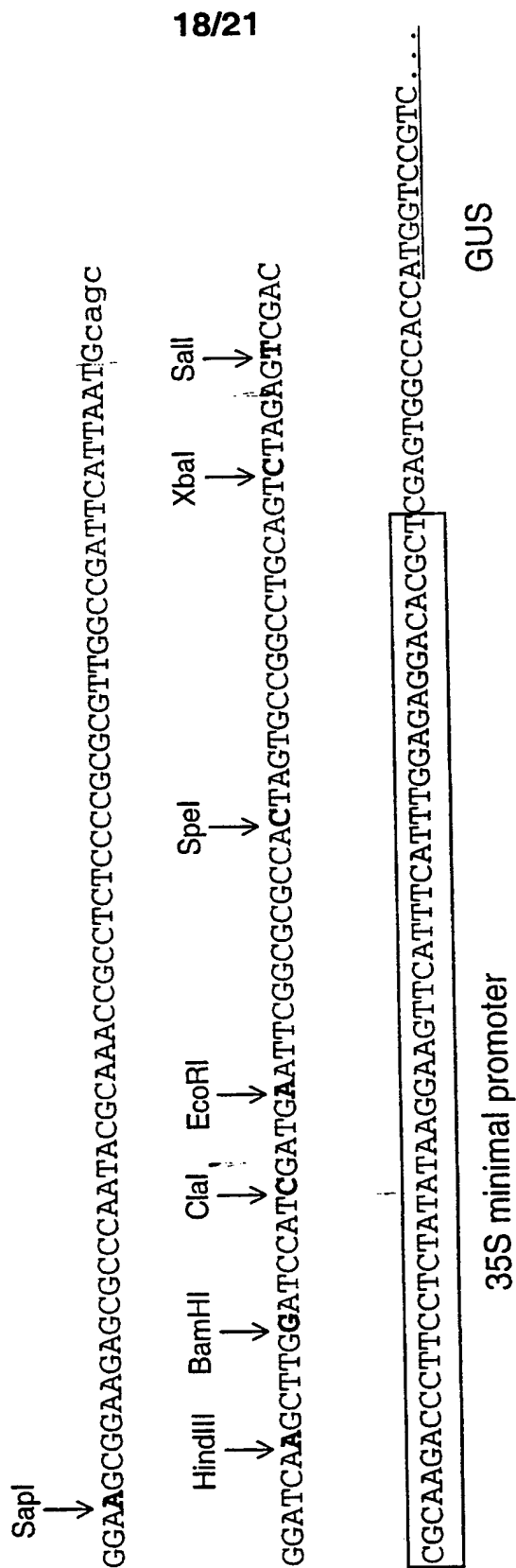


Fig. 6

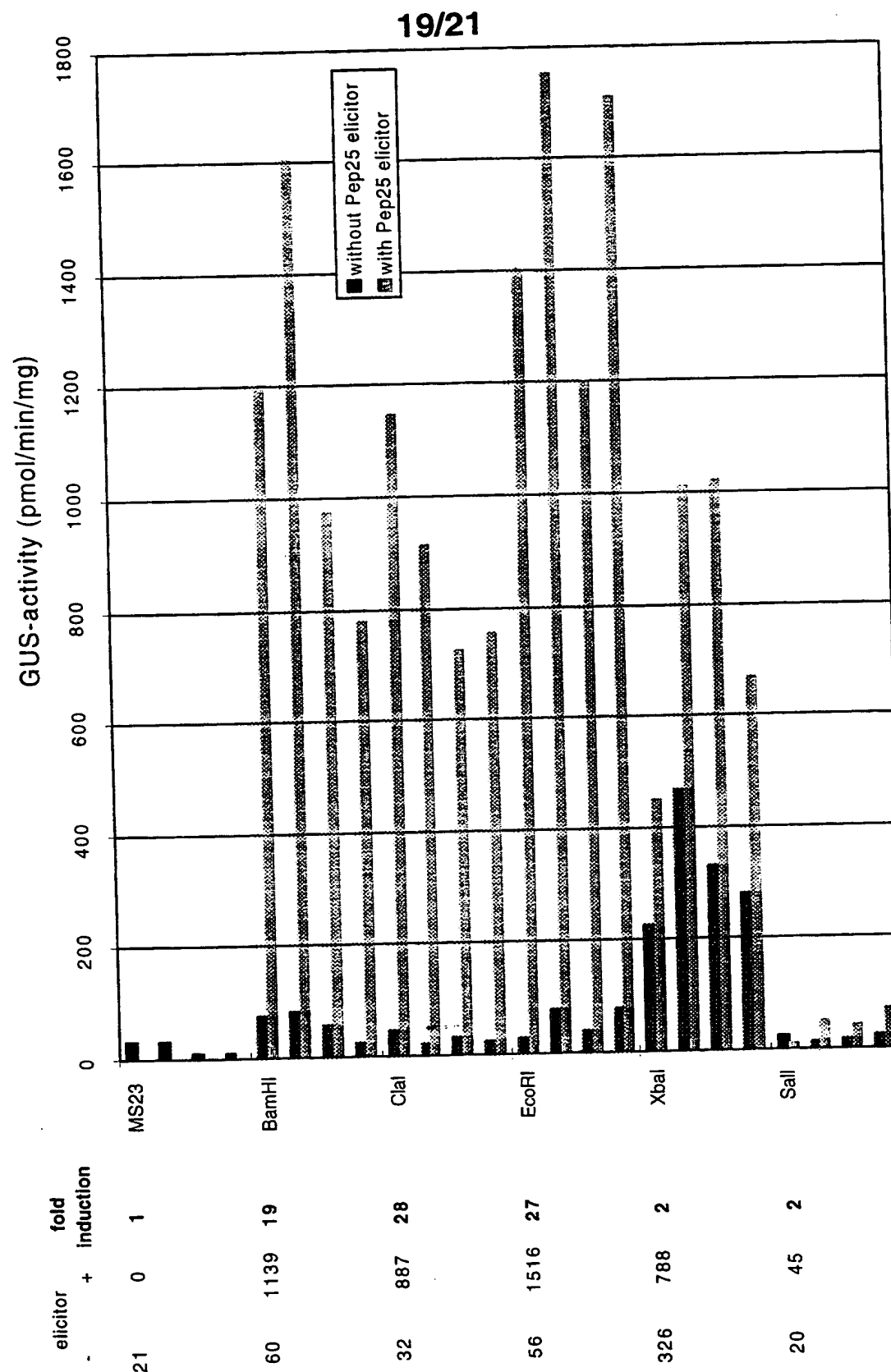


Fig. 7a

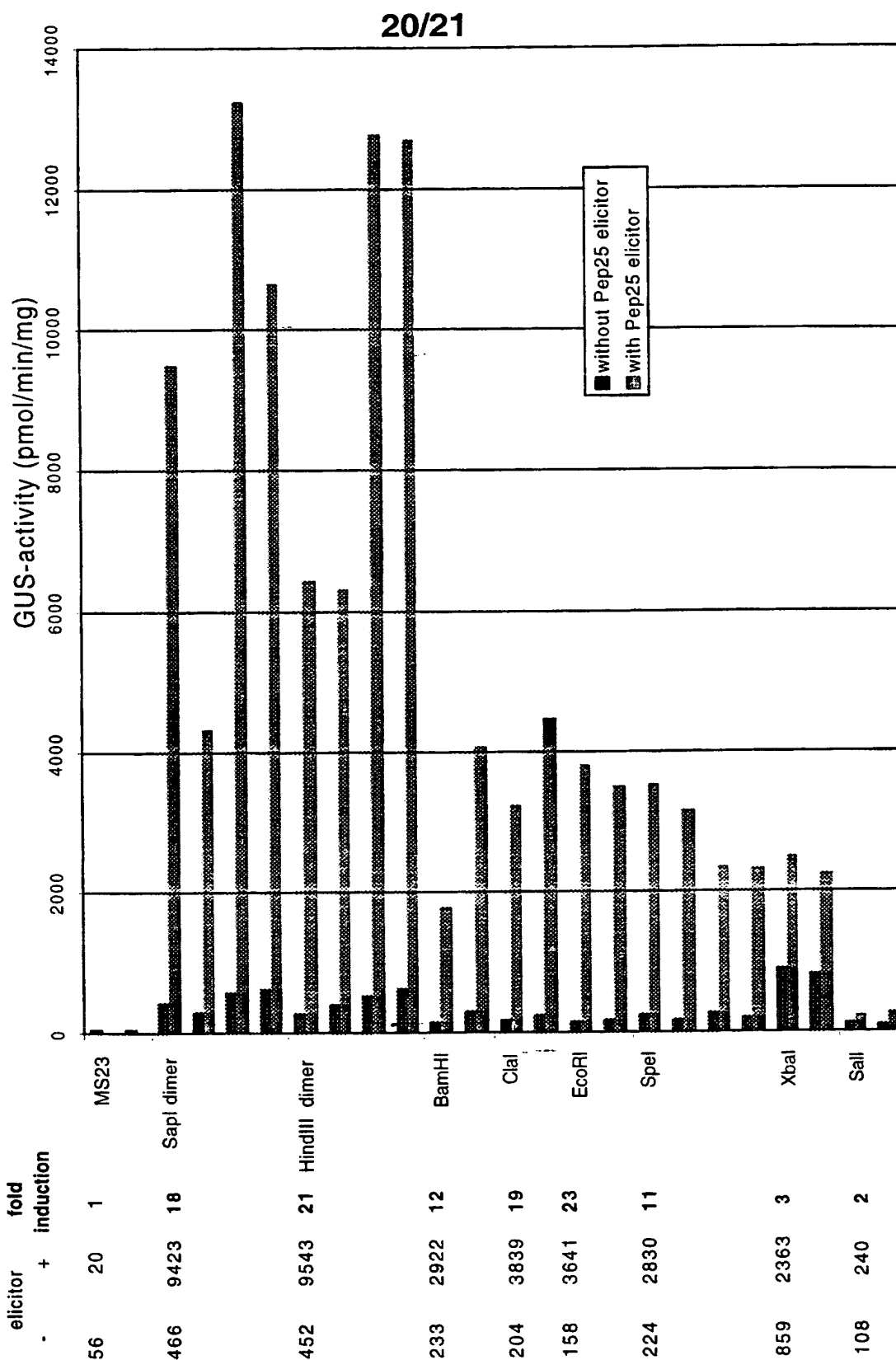


Fig. 7b

Summary of Expression Characteristics

	Aerial parts	Roots	Wounding	Senescence	<i>Peronospora</i>	
					Incompatible	Compatible
4 x S	Medium	Medium	+	nt	+	+
4 x W2	Medium	Very high	+	nt	+	+
4 x GCC	Very high	Very high	+	nt	nt	nt
4 x D	-	-	-	+	+	+
4 x N	Medium	Medium	+	nt	+	+
4 x W Amy	Low	Low	nt	nt	+	+
4 x W1	Medium	Medium	+	nt	+	+

Fig. 8

SEQUENCE LISTING

<110> Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

<120> Chimeric promoters capable of mediating local gene-expression in plants upon pathogen infection and uses thereof

<130> C 1078 PCT

<140>

<141>

<160> 17

<170> PatentIn Ver. 2.1

<210> 1

<211> 7

<212> DNA

<213> Petroselinum crispum

<400> 1

tttgacc

7

<210> 2

<211> 30

<212> DNA

<213> Petroselinum crispum

<400> 2

cacacttaat ttgaccgagt aacattcgcc

30

<210> 3

<211> 20

<212> DNA

<213> Petroselinum crispum

<400> 3

ttcagccnnn nnnnttgacc

20

<210> 4

<211> 29

<212> DNA

<213> Petroselinum crispum

<400> 4

ttattcagcc atcaaagttg accaataat

29

<210> 5

<211> 14

<212> DNA

<213> Triticum aestivum

<400> 5

tgacnnnnnn gtca

14

<210> 6

<211> 14

<212> DNA

<213> Triticum aestivum

<400> 6

tgacttgacc gtca	14
<210> 7	
<211> 24	
<212> DNA	
<213> Petroselinum crispum	
<400> 7	
cagccaccaa agaggaccca gaat	24
<210> 8	
<211> 16	
<212> DNA	
<213> Solanum tuberosum	
<400> 8	
gccaccnnnt ttgacc	16
<210> 9	
<211> 25	
<212> DNA	
<213> Solanum tuberosum	
<400> 9	
ttctagccac cagatttgac caaac	25
<210> 10	
<211> 24	
<212> DNA	
<213> Triticum aestivum	
<400> 10	
ggattgactt gaccgtcatc ggct	24
<210> 11	
<211> 31	
<212> DNA	
<213> Petroselinum crispum	
<400> 11	
tacaattcaa acattgttca aacaaggaac c	31
<210> 12	
<211> 15	
<212> DNA	
<213> Petroselinum crispum	
<400> 12	
agttgaaatt caata	15
<210> 13	
<211> 30	
<212> DNA	
<213> Petroselinum crispum	
<400> 13	
agttgaaatt caataagttg aaattcaata	30
<210> 14	

<211> 10
<212> DNA
<213> Petroselinum crispum

<400> 14
agccaccana

10

<210> 15
<211> 27
<212> DNA
<213> Petroselinum crispum

<400> 15
tcaatatgtc aatgggtcaac attcaac

27

<210> 16
<211> 11
<212> DNA
<213> Petroselinum crispum

<400> 16
tgtcaatggt caacattcaa c

21

<210> 17
<211> 4299
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic, no
natural origin

<400> 17
aggtggcact tttcgggggaa atgtgcgcgg aacccctatt tgtttatatt tctaaataca 60
ttcaaataatg tatccgctca tgagacaata accctgataa atgcttcaat aatattgaaa 120
aaggaagagt atgagtattc aacattttccg tgtcgccctt attccctttt ttgcggcatt 180
ttgccttccct gttttttgctc acccagaaac gctgggtgaaa gtaaaagatg ctgaagatca 240
gttgggtgca cgagtgggtt acatcgaact ggatctcaac agcggtaaga tccttgagag 300
ttttcgcccc gaagaacgtt ttccaatgat gagcactttt aaagtctctgc tatgtggcgc 360
ggattattcc cgtattgacg cggggcaaga gcaactcggc cgccgcatac actattctca 420
gaatgacttg gttgagtact caccagtcac agaaaagcat cttacggatg gcatgacagt 480
aagagaatta tgcagtgtcg ccataaccat gagtgataac actgcggccca acttacttct 540
gacaacgatc ggaggaccga aggagctaac cgcttttttg cacaacatgg gggatcatgt 600
aactgcctt gatcgttggg aaccggagct gaatgaagcc ataccaaacg acgagcgtga 660
caccacgatg cctgttagcaa tggcaacaac gttgcgcaaa ctattaactg gcgaactact 720
tactctagct tcccggcaac aattaataga ctggatggag gcggtataaag ttgcaggacc 780
acttctgcgc tcggcccttc cggctggctg gtttattgct gataaatctg gagccggtga 840
gcgtgggtct cgcggtatca ttgcagcact gggggccagat ggtaagccct cccgtatcgt 900
agttatctac acgacgggga gtcaggcaac tatggatgaa cgaaatagac agatcgctga 960
gataggtgcc tcaactgatta agcattggta actgtcagac caagtttact catatatact 1020
ttagattgat ttaaaacttc atttttaatt taaaaggatc taggtgaaga tcctttttga 1080
taatctcatg accaaaatcc cttaacgtga gttttcgttc cactgagcgt cagaccccggt 1140
agaaaagatc aaaggatctt cttgagatcc tttttttctg cgcgtaatct gctgcttgca 1200
aacaataaaa ccaccgtac cagcgtggt ttgtttgccc gatcaagagc taccactct 1260
ttttccgaag gtaactggct tcagcagagc gcagatacca aatactgtcc ttctagtgtg 1320
gccgtagtta ggccaccact tcaagaactc tgtagcaccg cctacatacc tcgctctgct 1380
aatcctgtta ccagtggctg ctgccagtgg cgataagtcg tgtcttaccg ggttggactc 1440
aagacgatag ttaccgata aggcgcagcg gtcgggctga acgggggggt cgtgcacaca 1500
gccagcttg gagcgaacga cctacaccga actgagatac ctacagcgtg agcattgaga 1560
aagcgccacg cttcccgaag ggagaaaggc ggacaggat cgggtaagcg gcagggtcgg 1620
aacaggagag cgcacgaggg agcttccagg gggaaacgcc tgggtatctt atagtcctgt 1680
cgggtttcgc cacctctgac ttgagcgtcg atttttgtga tgctcgtcag gggggcggag 1740
cctatggaaa aacgccagca acgcggcctt ttacaggttc ctggcctttt gctggccttt 1800
tgctcacatg ttctttctcg cgttatcccc tgattctgtg gataaccgta ttaccgcctt 1860

tgagtgaagt	gataccgctc	gccgcagccg	aacgaccgag	cgcagcgagt	cagtgagega	1920
ggaagcgga	gagcgccaa	tacgcaaacc	gcctctcccc	gcgcgttggc	cgattcatta	1980
atgcagcgga	tcaagcttgg	atccatcgat	gaattcggcg	cgccactagt	gccggcctgc	2040
agtctagagt	cgaccgcaag	acccttcctc	tatataagga	agttcatttc	atttggagag	2100
gacacgctcg	agtggccacc	atggtccgtc	ctgtagaaac	cccaaccctg	gaaatcaaaa	2160
aactcgacgg	cctgtgggca	ttcagtctgg	atcgcgaaaa	ctgtggaatt	gatcagcggt	2220
ggtgggaaag	cgcggttaca	gaaagccggg	caattgctgt	gccaggcagt	tttaacgata	2280
agttcgccga	tgcagatatt	cgtaattatg	cgggcaacgt	ctggatcag	cgcgaaatct	2340
ttataccgaa	aggttgggca	ggccagcgta	tcgtgctgcg	tttcgatgcg	gtcactcatt	2400
acggcaaagt	gtgggtcaat	aatcaggaag	tgatggagca	tcaggcgggc	tatacgccat	2460
ttgaagccga	tgtcacgccc	tatgttattg	ccgggaaaaa	tgtacgtatc	accgtttgtg	2520
tgaacaacga	actgaactgg	cagactaatg	cgccgggaat	ggtgattacc	gacgaaaaacg	2580
gcaagaaaaa	gcagtcttac	ttccatgatt	tctttaacta	tgccggaatc	catcgacgag	2640
taatgtctta	caccacgccc	aacacctggg	tggaacgata	caccgtgggtg	acgcattgtcg	2700
cgcaagactg	taaccacgcg	tctgttgact	ggcaggtggg	ggccaatggg	gatgtcagcg	2760
ttgaactcga	tgatgaggat	caacaggtgg	ttgcaactgg	acaaggcact	agcgggactt	2820
tgcaagtggg	gaatcgccac	ctctggcaac	cggttgaagg	ttatctctat	gaactgtgag	2880
tcacagccaa	aagccagaca	gagtgtgata	tctaccgctc	tcgcgtcgcc	atccggtcag	2940
tggaagtgaa	gggcaaacag	ttcctgatta	accacaaacc	gttctacttt	actggctttg	3000
gtcgtcatga	agatgaggac	ttgcgtggca	aaggattcga	taacgtgctg	atggtgcacg	3060
accacgcatt	aatggactgg	attggggcca	actcctaccg	tacctcgcat	taccttaccg	3120
ctgaagagat	gctcgactgg	gcagatgaac	atggcatcgt	ggtgattgat	gaaactgctg	3180
ctgtcggctt	taacctctct	ttaggcattg	gtttcgaagc	gggcaacaag	ccgaaagaac	3240
tgtacagcga	agaggcagtc	aacggggaaa	ctcagcaagc	gcacttacag	gcgattaaag	3300
agctgatagc	gcgtgacaaa	aaccacccaa	gcgtggtgat	gtggagtatt	gccaacgaac	3360
cggatacccg	tccgcaagg	gcacgggaat	atttcgcgcc	actggcgga	gcaacgcgta	3420
aactcgaccc	gacgcgtccg	atcacctgag	tcaatgtaat	gttctgcgac	gctcacaccg	3480
ataccatcag	cgatctcttt	gatgtgctgt	gcctgaaccg	ttattacgga	tggtatgtcc	3540
aaagcggcga	tttggaaacg	gcagagaagg	tactggaaaa	agaacttctg	gcctggcagg	3600
agaaactgca	tcagccgatt	atcatcaccg	aatacggcgt	ggatacgtta	gccgggctgc	3660
actcaatgta	caccgacatg	tggaagtgaag	agtatcagtg	tgcatggctg	gatattgata	3720
accgcgtctt	tgatcgcgct	agcgccgctg	tcggtgaaca	ggtatggaat	ttcgccgatt	3780
ttgcgacctc	gcaaggcata	ttgcgcgttg	gcggtaacaa	gaaagggatc	ttcactcgcg	3840
accgcaaaac	gaagtccggc	gcttttctgc	tgcaaaaacg	ctggactggc	atgaacttcg	3900
gtgaaaaacc	gcagcagggg	ggcaaaaca	gaatcaacaa	ctctcctggc	gcaccatcgt	3960
cgctacagcc	tcgggaattg	ctaccgagct	ccgggttacc	tgatcatgag	taattagctc	4020
gaatttcccc	gatcgttcaa	acatttggca	ataaagtttc	ttaagattga	atcctgttgc	4080
cggtcttgcg	atgattatca	tataatttct	gttgaattac	gttaagcatg	taataattaa	4140
catgtaatgc	atgacgttat	ttatgagatg	ggtttttatg	attagagtcc	cgcaattata	4200
catttaatac	gcgatagaaa	acaaaatata	gcgcgcaaac	taggataaat	tatcgcgccg	4260
ggtgtcatct	atgttactag	atcggaat	agatctgct			4299

REC'D 28 FEB 2001

WIPO

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference C 1078 PCT		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP99/08710	International filing date (day/month/year) 12/11/1999	Priority date (day/month/year) 12/11/1998
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant MAX-PLANCK-GESELLSCHAFT ...et.al		



1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 27/04/2000	Date of completion of this report 26.02.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Huber, A Telephone No. +49 89 2399 8173 

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/08710

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1-41 as originally filed

Claims, No.:

1-40 as received on 01/02/2001 with letter of 01/02/2001

Drawings, sheets:

1/21-21/21 as originally filed

Sequence listing part of the description, pages:

1-4, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/08710

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
☒ claims Nos. 2, 28, 32, 40 (completely), 3-27, 29-31, 33-34, 37-39 (partially).

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 2, 28, 32, 40 (completely); 3-27, 29-31, 33-34, 37-39 (partially).
2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/08710

citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1, 3-27, 29-31, 37
	No:	Claims	33-36, 38, 39
Inventive step (IS)	Yes:	Claims	1, 3-27, 29-31, 37
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-40
	No:	Claims	

**2. Citations and explanations
see separate sheet**

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Due to the finding of lack of unity of invention, the international search was restricted to promoters comprising cis-acting elements comprising the nucleotide sequences shown in SEQ ID Nos. 3 or 4. Consequently, preliminary examination is carried out only for those embodiments for which a search report has been established, i.e. to promoters comprising a cis-acting element having the nucleotide sequence of SEQ ID Nos. 3 or 4.
In the amended set of claims Claim 1 has been restricted to a chimeric promoter comprising at least two cis-acting elements comprising the nucleotide sequence of SEQ ID Nos. 3 or 4.
Claim 2 relates to a chimeric promoter comprising at least one cis-acting element comprising the nucleotide sequence of any of SEQ ID Nos. 5-16.
Since no search has been performed for the subject-matter of Claim 2, no preliminary examination can be carried out for said claim and for claims dependent thereon.
2. In addition, no search has been performed for the subject-matter of original Claims 27, 31 and 39, now Claims 28, 32 and 40 and only a partial search has been performed of original Claims 28-30 and 32 (now Claims 29-31 and 33), since said claims relate to purely speculative compounds (activators or inhibitors of gene expression), which are not sufficiently disclosed and supported in the application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present application relates to chimeric promoters capable of mediating local gene expression in plants upon pathogen infection comprising a cis-acting

element and a minimal promoter.

2. Reference is made to the following documents:

D1: RUSHTON P J ET AL.: 'Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes' EMBO JOURNAL, vol. 15, no. 20, 1996, pages 5690-5700, XP002138284 cited in the application

D2: WO-A-95/03690

D3: WO-A-96/36697

D4: WO-A- 98/ 03536

D5: WO-A-96/28561

D6: RAVENTOS D ET AL.: 'A 20 bp cis-acting element is both necessary and sufficient to mediate elicitor response of a maize PRms gene' PLANT JOURNAL, vol. 7, no. 1, January 1995 (1995-01), pages 147-155, XP002107426 cited in the application

3. In **D1** fungal elicitor responsive elements (boxes W1, W2 and W3) have been identified as a cis-acting elements which are required for elicitor responsive expression of parsley PR1 promoters in a transient expression system in plant protoplasts. Box W2 comprises the nucleotide sequence shown in SEQ ID Nos. 3 and 4. A promoter construct comprising a cis-acting element comprising the nucleotide sequence shown in SEQ ID No.3 and 4 and a minimal promoter has been disclosed in **D1** (see e.g. Fig. 1, construct PR1-1h). Also promoter constructs comprising box W1 and box W2 are disclosed in **D1** (see Fig. 1). Box W1 comprises the nucleotide sequence of SEQ ID Nos. 1 and 2. The capability of mediating local gene expression in plants upon pathogen infection has to be considered as an inherent property of the construct of **D1**.

In **D1** and in **D6** it has further been shown that multiple copies of elicitor response elements (box W1 of the Pr1-promoter and ERE from PRms promoter) enhance elicitor responsiveness (see **D1**, p. 5693, Fig. 5 and first full paragraph on p. 5693; **D6**, p. 150 to p. 151, first paragraph, Fig. 5). In addition, in Fig. 1 of **D1** it has already been shown that the W2 box alone has stronger elicitor inducible activity than the W1 box. These results would prompt the skilled person to use more than one copy of a further ERE known from **D1** (W2 box). However, the use of a spacer region composed of 4-10 base pairs to separate at least two of said elements has neither been disclosed nor suggested in **D1** or **D6**.

Therefore, the introduction of the additional features that the chimeric promoter comprises **at least two** cis-acting elements which comprise the nucleotide sequence of SEQ ID Nos. 3 or 4 and wherein **a spacer region** separates at least two of said cis-acting elements render the subject-matter of amended Claim 1 and the thereon dependent claims novel and inventive over the disclosure of **D1** (Art. 33(2) and (3) PCT).

4. Independent Claim 35 is directed to a general method of rendering a gene responsive to pathogens by inserting at least one cis-acting element into the promoter of said gene. Independent Claim 36 concerns a general method for preparing a promoter comprising operably linking a cis-acting regulatory element to a transcription initiation sequence of a promoter. Claims 33 and 34 embrace in a first embodiment the use of a(ny) cis-acting element sufficient to direct elicitor-specific expression for the production of pathogen resistant plants, resp. for identifying and/or producing compounds capable of conferring induced resistance to a pathogen in a plant.

The subject-matter of said claims could be regarded as representing separate inventions since it does not relate to the **specific** cis-acting elements capable of directing elicitor-specific expression in plant cells referred to in Claims 1-32. However, no objection to lack of unity has been raised with regard to said claims by the search authority. Instead, several documents have been cited which destroy the novelty of said claims.

D2, for example, discloses the preparation of a chimeric promoter, wherein a cis-acting regulatory element of a HMG2 promoter is inserted into a plant promoter thereby rendering the gene linked to said chimeric promoter responsive to a

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP99/08710

pathogen (see D2, p. 16, l. 8-19, p. 39, l. 18-30, claims).

Also **D3** is directed to the insertion of an inducible regulatory element into a gene which mediates the expression of the gene upon induction by a plant pathogen. Similarly, **D4** discloses a chemically inducible chimeric promoter comprising a regulatory element derived from the Arabidopsis Pr-1 promoter, the chimeric promoter driving controlled expression of e.g. genes providing resistance to plant pests.

D5 concerns a fungus-responsive promoter which comprise a fungus-responsive prp-1 element. The promoter mediates local gene expression in plants at the site of fungal infection.

D6 discloses cis-acting sequences which, when inserted into the PRms promoter, confer localized elicitor-responsive expression on the maize PRms gene in plant cells upon pathogen infection.

Hence, any of these documents destroys the novelty of Claims 33-36, 38 and 39.

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
 United States Patent and Trademark
 Office
 Box PCT
 Washington, D.C.20231
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 27 July 2000 (27.07.00)	
International application No. PCT/EP99/08710	Applicant's or agent's file reference C 1078 PCT
International filing date (day/month/year) 12 November 1999 (12.11.99)	Priority date (day/month/year) 12 November 1998 (12.11.98)
Applicant KIRSCH, Christoph et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
 27 April 2000 (27.04.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer F. Baechler Telephone No.: (41-22) 338.83.38
--	--

INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/EP 99/08710

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N5/10 C07K16/16 G01N33/50 A01H5/00
A01N63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUSHTON P J ET AL.: "Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes" EMBO JOURNAL, vol. 15, no. 20, 1996, pages 5690-5700, XP002138284 cited in the application the whole document ---	1-7, 9-11, 13-16, 21-26, 29,30, 34-38
X	WO 98 03536 A (LEBEL EDOUARD GUILLAUME ;RYALS JOHN ANDREW (US); WARD ERIC RUSSELL) 29 January 1998 (1998-01-29) the whole document ---	34,35, 37,38
X	WO 95 03690 A (VIRGINIA TECH INTELL PROP) 9 February 1995 (1995-02-09) the whole document ---	34,35, 37,38
	--- -/--	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

22 May 2000

Date of mailing of the international search report

28. 08. 00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

INTERNATIONAL SEARCH REPORT

Inter Application No

PC1/EP 99/08710

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 36697 A (UNIV KENTUCKY) 21 November 1996 (1996-11-21) the whole document ---	34,35, 37,38
X	WO 96 28561 A (MAX PLANCK GESELLSCHAFT ;STRITTMATTER GUENTER (DE)) 19 September 1996 (1996-09-19) the whole document ---	34,35, 37,38
X	RAVENTOS D ET AL.: "A 20 bp cis-acting element is both necessary and sufficient to mediate elicitor response of a maize PRms gene" PLANT JOURNAL, vol. 7, no. 1, January 1995 (1995-01), pages 147-155, XP002107426 cited in the application the whole document -----	34,35, 37,38

INTERNATIONAL SEARCH REPORT

I. Information on patent family members

International Application No

PCT/EP 99/08710

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9803536 A	29-01-1998	AU 708850 B AU 3804897 A CA 2232741 A EP 0868426 A JP 11513897 T	12-08-1999 10-02-1998 29-01-1998 07-10-1998 30-11-1999
WO 9503690 A	09-02-1995	AU 7518994 A CA 2168430 A EP 0712273 A JP 9503652 T SG 46678 A US 5670349 A US 5689056 A ZA 9405745 A	28-02-1995 09-02-1995 22-05-1996 15-04-1997 20-02-1998 23-09-1997 18-11-1997 14-03-1995
WO 9636697 A	21-11-1996	US 5981843 A AU 5732196 A BG 102047 A BR 9602338 A CA 2221348 A CN 1191565 A CZ 9703656 A EP 0828822 A ES 2134170 A HU 9802383 A JP 11505423 T PL 323383 A ZA 9603957 A	09-11-1999 29-11-1996 30-11-1998 13-01-1998 21-11-1996 26-08-1998 17-06-1998 18-03-1998 16-09-1999 28-01-1999 21-05-1999 30-03-1998 25-11-1996
WO 9628561 A	19-09-1996	AU 716814 B AU 1949695 A EP 0759085 A JP 10500312 T US 5910629 A	09-03-2000 02-10-1996 26-02-1997 13-01-1998 08-06-1999

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/EP 99/08710

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: {27, 31, 39 complete}; {28, 29, 30, 32 partially}
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-39 all partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: {1-39 all partially}

A chimeric promoter capable of mediating local gene expression in plants upon pathogen infection comprising at least one cis-acting element sufficient to direct elicitor-specific expression comprising the nucleotide sequence of SEQ ID NO: 3 or 4, and a minimal promoter. A recombinant gene, a vector, a method for the production of transgenic plants, plant cells, a transgenic plant, a cis-acting element, a method for the identification of an activator or inhibitor, an antibody, a diagnostic composition, a kit comprising said nucleotide sequence.

2. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 5 or 6.

3. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 7.

4. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 8 or 9.

5. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 10.

6. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 11.

7. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 12 or 13.

8. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 14.

9. Claims: {1-39 all partially}

same as invention 1 but comprising SEQ ID NO: 15 or 16.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: {27, 31, 39 complete}; {28, 29, 30, 32 partially}

Claims 27-32 and 39 refer to an activator or inhibitor of the polypeptide without giving a true technical characterization. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum)

C 1078 PCT

Box No. I TITLE OF INVENTION

Chimeric promoters capable of mediating gene expression in plants upon pathogen infection and uses thereof

Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

Max-Planck-Gesellschaft zur Förderung der
Wissenschaften e.V.
Berlin
DE

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State (that is, country) of nationality:

DE

State (that is, country) of residence:

DE

This person is applicant
for the purposes of:☐ all designated
States☒ all designated States except
the United States of America☐ the United States
of America only☐ the States indicated in
the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

KIRSCH, Christoph
Am Schulberg 9
50858 KÖLN
DE

This person is:

☐ applicant only☒ applicant and inventor☐ inventor only (If this check-box
is marked, do not fill in below.)

State (that is, country) of nationality:

DE

State (that is, country) of residence:

DE

This person is applicant
for the purposes of:☐ all designated
States☐ all designated States except
the United States of America☒ the United States
of America only☐ the States indicated in
the Supplemental Box☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Vossius & Partner (No. 31)
Siebertstrasse 4
81675 München
DE

Telephone No.

49-89-413 040

Facsimile No.

49-89-413 04 111

Teleprinter No.

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

LOGEMANN, Elke
An der Fuchskaul 2
50259 Pulheim-Dansweiler
DE

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
DE

State (that is, country) of residence:
DE

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

HAHLBROCK, Klaus
Bachstr. 8a
50858 Köln
DE

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
DE

State (that is, country) of residence:
DE

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

RUSHTON, Paul
24, Poplar Road
Botley, Oxford OX2 9LB
UK

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
UK

State (that is, country) of residence:
UK

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

SOMSSICH, Imre
Kolibriweg 2
50829 Köln
DE

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
US

State (that is, country) of residence:
DE

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- | | |
|--|--|
| <input checked="" type="checkbox"/> AE United Arab Emirates | <input checked="" type="checkbox"/> LR Liberia |
| <input checked="" type="checkbox"/> AL Albania | <input checked="" type="checkbox"/> LS Lesotho |
| <input checked="" type="checkbox"/> AM Armenia | <input checked="" type="checkbox"/> LT Lithuania |
| <input checked="" type="checkbox"/> AT Austria | <input checked="" type="checkbox"/> LU Luxembourg |
| <input checked="" type="checkbox"/> AU Australia | <input checked="" type="checkbox"/> LV Latvia |
| <input checked="" type="checkbox"/> AZ Azerbaijan | <input checked="" type="checkbox"/> MD Republic of Moldova |
| <input checked="" type="checkbox"/> BA Bosnia and Herzegovina | <input checked="" type="checkbox"/> MG Madagascar |
| <input checked="" type="checkbox"/> BB Barbados | <input checked="" type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input checked="" type="checkbox"/> BG Bulgaria | |
| <input checked="" type="checkbox"/> BR Brazil | <input checked="" type="checkbox"/> MN Mongolia |
| <input checked="" type="checkbox"/> BY Belarus | <input checked="" type="checkbox"/> MW Malawi |
| <input checked="" type="checkbox"/> CA Canada | <input checked="" type="checkbox"/> MX Mexico |
| <input checked="" type="checkbox"/> CH and LI Switzerland and Liechtenstein | <input checked="" type="checkbox"/> NO Norway |
| <input checked="" type="checkbox"/> CN China | <input checked="" type="checkbox"/> NZ New Zealand |
| <input checked="" type="checkbox"/> CU Cuba | <input checked="" type="checkbox"/> PL Poland |
| <input checked="" type="checkbox"/> CZ Czech Republic | <input checked="" type="checkbox"/> PT Portugal |
| <input checked="" type="checkbox"/> DE Germany | <input checked="" type="checkbox"/> RO Romania |
| <input checked="" type="checkbox"/> DK Denmark | <input checked="" type="checkbox"/> RU Russian Federation |
| <input checked="" type="checkbox"/> EE Estonia | <input checked="" type="checkbox"/> SD Sudan |
| <input checked="" type="checkbox"/> ES Spain | <input checked="" type="checkbox"/> SE Sweden |
| <input checked="" type="checkbox"/> FI Finland | <input checked="" type="checkbox"/> SG Singapore |
| <input checked="" type="checkbox"/> GB United Kingdom | <input checked="" type="checkbox"/> SI Slovenia |
| <input checked="" type="checkbox"/> GD Grenada | <input checked="" type="checkbox"/> SK Slovakia |
| <input checked="" type="checkbox"/> GE Georgia | <input checked="" type="checkbox"/> SL Sierra Leone |
| <input checked="" type="checkbox"/> GH Ghana | <input checked="" type="checkbox"/> TJ Tajikistan |
| <input checked="" type="checkbox"/> GM Gambia | <input checked="" type="checkbox"/> TM Turkmenistan |
| <input checked="" type="checkbox"/> HR Croatia | <input checked="" type="checkbox"/> TR Turkey |
| <input checked="" type="checkbox"/> HU Hungary | <input checked="" type="checkbox"/> TT Trinidad and Tobago |
| <input checked="" type="checkbox"/> ID Indonesia | <input checked="" type="checkbox"/> UA Ukraine |
| <input checked="" type="checkbox"/> IL Israel | <input checked="" type="checkbox"/> UG Uganda |
| <input checked="" type="checkbox"/> IN India | <input checked="" type="checkbox"/> US United States of America |
| <input checked="" type="checkbox"/> IS Iceland | |
| <input checked="" type="checkbox"/> JP Japan | <input checked="" type="checkbox"/> UZ Uzbekistan |
| <input checked="" type="checkbox"/> KE Kenya | <input checked="" type="checkbox"/> VN Viet Nam |
| <input checked="" type="checkbox"/> KG Kyrgyzstan | <input checked="" type="checkbox"/> YU Yugoslavia |
| <input checked="" type="checkbox"/> KP Democratic People's Republic of Korea | <input checked="" type="checkbox"/> ZA South Africa |
| | <input checked="" type="checkbox"/> ZW Zimbabwe |
| <input checked="" type="checkbox"/> KR Republic of Korea | Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet: |
| <input checked="" type="checkbox"/> KZ Kazakhstan | <input checked="" type="checkbox"/> CR Costa Rica |
| <input checked="" type="checkbox"/> LC Saint Lucia | <input checked="" type="checkbox"/> TZ Tanzania |
| <input checked="" type="checkbox"/> LK Sri Lanka | <input checked="" type="checkbox"/> DM Dominica |
| | <input checked="" type="checkbox"/> MA Morocco |

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Box No. VI PRIORITY CLAIM		<input type="checkbox"/> Further priority claim indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: regional Office	international application: receiving Office
item (1) 12/11/98 12 Nov. 1998	98 12 1160.0		EP	
item (2) 27/08/99 27 Aug. 1999	99 11 6981.4		EP	
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): **1 and 2**

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA)
(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA /

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year)

Number

Country (or regional Office)

Box No. VIII CHECK LIST: LANGUAGE OF FILING

This international application contains the following number of sheets:

request : 4

description (excluding sequence listing part) : 41

claims : 5

abstract : 1

drawings : 21

sequence listing part of description : 4

Total number of sheets : 76

This international application is accompanied by the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other (specify):

Figure of the drawings which should accompany the abstract:

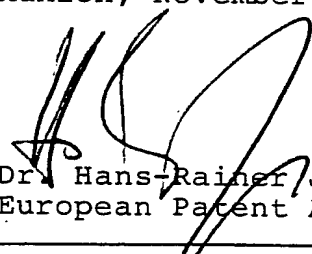
Language of filing of the international application: **ENGLISH**

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request):

Munich, November 12, 1999

Jae/OM/mb


Dr. Hans-Rainer Jaenichen
European Patent Attorney

VOSSIUS & PARTNER
PATENTANWÄLTE
SIEBERTSTR. 4
81675 MÜNCHEN
(No. 31)

For receiving Office use only		2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received:
1. Date of actual receipt of the purported international application:		
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority (if two or more are competent): ISA /	6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.	

Date of receipt of the record copy by the International Bureau: